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FINAL REPORT

# "MOLECULAR MECHANISMS OF CYTOPATHOGENICITY OF PRIMATE LYMPHOTROPIC RETROVIRUSES: RELEVANCE TO TREATMENT AND VACCINE FOR AIDS"



BIOTECH RESEARCH LABORATORIES, INC.
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FINAL REPORT

By:
MARK M. MANAK AND LINDA L. JAGODZINSKI

AUGUST 10, 1990

Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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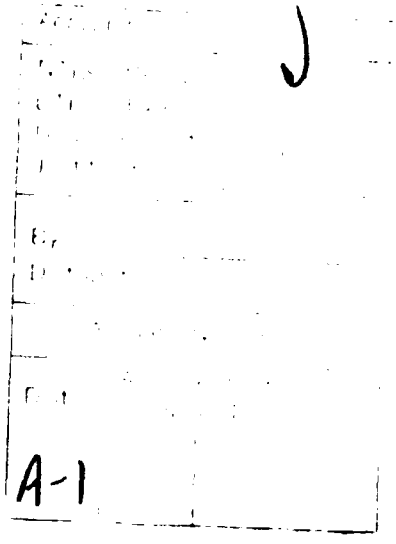
Rockville, Maryland 20850



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Studies were designed to examine the molecular basis of infectivity, cytopathogenicity and genomic activation of HIV-1, with the particular emphasis on examining the factors relevant to the design of treatment and vaccine programs for combating HIV-1 infection. Three parallel approaches were followed in the course of this work. The results of this work can be summarized as follows:

1. In order to obtain a better understanding of viral gene function involved in cell killing, a series of HIV-1 viral deletion mutants were generated and the biological consequences of these mutations were examined by transfection into recipient host cells. Our results have shown that mutations in vif result in the production of morphologically normal virus particles, but which are deficient in cell-free (but not cell-to-cell) transmission to permissive cells. Mutations in the carboxy region of gp41 result in dramatic changes in the cell-to-cell transmission and cytopathic properties of the virus. Mutations in the 5' region result in packaging defective genomes which lead to the formation of morphologically normal viral particles which are devoid of viral RNA and exhibit diminished infectivity.


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# "MOLECULAR MECHANISMS OF CYTOPATHOGENICITY OF PRIMATE LYMPHOTROPIC RETROVIRUSES: RELEVANCE TO TREATMENT AND VACCINE FOR AIDS"

FINAL REPORT

Covering the Period of:

SEPTEMBER 29, 1986 THROUGH DECEMBER 28, 1989

By:
MARK M. MANAK AND LINDA L. JAGODZINSKI

AUGUST 10, 1990

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# SUMMARY

Studies were designed to examine the molecular basis of infectivity, cytopathogenicity and genomic activation of HIV-1, with the particular emphasis on examining the factors relevant to the design of treatment and vaccine programs for combating HIV-1 infection. Three parallel approaches were followed in the course of this work. The results of this work can be summarized as follows:

1. In order to obtain a better understanding of viral gene function involved in cell killing, a series of HIV-1 viral deletion mutants were generated and the biological consequences of these mutations were examined by transfection into recipient host cells. Our results have shown that mutations in vif result in the production of morphologically normal virus particles, but which are deficient in cell-free (but not cell-to-cell) transmission to permissive cells. Mutations in the carboxy region of gp41 result in dramatic changes in the cell-to-cell transmission and cytopathic properties of the virus. Mutations in the 5' region result in packaging defective genomes which lead to the formation of morphologically normal viral particles which are devoid of viral RNA and exhibit diminished infectivity.

2. The molecular mechanism of transactivation by Tat was examined to elucidate the events that trigger the transition from a low level latent infection to productive HIV-1 replication. Mutation studies have identified an enchancer element, an Sp-1 binding site, and a tat response region (TAR) within the HIV-1 LTR. Additional studies of Tat activity demonstrated direct binding of Tat to TAR RNA, a synergistic stimulation of LTR by Tat and mitogens, and both transcriptional and postranscriptional activation mechanisms by Tat.

3. The genome organization of several related retroviruses, including SIV and HIV-2 were examined to allow a comparison of the underlying differences in their cytopathicity, and to determine the suitability of these genomes for use in an animal model for AIDS. An HIV-2 isolate ($HIV\text{-}2_{SBL/ISY}$) capable of propagating in a variety of lymphotropic cell lines and infecting and killing cultured T-cells from Rhesus macaques was obtained and characterized. Rhesus macaques infected with this isolate were shown to be infected as detected by antibody production, cultivation of PBLs followed by Reverse Transcriptase activity, and by the Polymerase Chain Reaction Assay. This HIV-2 isolate may be suitable for use in an animal model for AIDS, and studies in characterizing this model have been initiated.

# FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these orgainizations.

In conducting the research described in this report, the investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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# I. BODY OF THE REPORT

## STATEMENT OF PROBLEM

Acquired Immune Deficiency Syndrome (AIDS) is a progressive, incurable disease, which attacks the victims' immune system leaving him vulnerable to a variety of opportunistic diseases which inevitably results in death. The causative agent of AIDS has been shown to be infection with the human immune deficiency virus (HIV) a human retrovirus which selectively infects, and eventually kills OKT4+ helper/inducer lymphocyte cells which play a critical role in the regulation of the immune response. In spite of the relatively small viral genome (approximately 10 kb), HIV-1 induces a variety of functions and complex viral-host interactions whose expression is tightly regulated. Initial infection with HIV-1 may produce no symptoms in the host as the virus enters a quiescent or dormant stage. The virus, however, can become reactivated up to several years after the initial infection, with devastating effects. The rapid spread of AIDS in the U.S. and worldwide has led to extensive efforts to develop therapy regimens for those already infected with HIV and to develop vaccines to protect populations at risk for the disease. A clear understanding of the biological complexities of HIV infections and the molecular basis of viral gene regulation, formation and transmission of viral particles, and cytopathogenicity is essential for designing systematic approaches to the development of appropriate vaccines and therapies for combating AIDS.

In the course of this contract, we examined the genomes of HIV-1 and related retroviruses and attempted to relate specific viral sequences to the functions they induce. The DNA sequence of a number of HIV-1, HIV-2 and SIV isolates was determined and analyzed in terms of its genome organization, coding capacity, and relationship to the other isolates. The biological parameters of infection were examined by using cloned viral DNA sequences to infect a cell culture system which provided an in vitro model for AIDS. Specific mutations were introduced in the non-structural portion of the viral genome and the consequences of these mutations examined in terms of the

biological expression upon infection into permissive host cells. The work carried out in the course of this contract was divided into 3 major sections.

Section 1: Cloned viral DNAs containing deletion mutations in various portions of the viral genome were used to transfect permissive cells. The formation of infectious virus particles, transmission to uninfected cells, and cell killing properties of the resulting mutant viruses were analyzed, allowing us to dissect the molecular basis of viral gene functions.

Section 2: The molecular mechanism of viral gene activation was examined at the level of LTR-tat interactions. Using a series of deletion mutations and cloned viral DNA constructs in in vitro model systems for activation, a variety of viral, cellular, and environmental factors which are involved in viral gene activation were examined.

Section 3: The DNA sequence of a number of isolates of HIV-2 and SIV was determined and compared to that of HIV-1. These differences were analyzed with respect to the observed differences in the biological properties of these isolates, particularly with respect to cytopathicity. The suitability of these isolates for use in animal models for AIDS was also considered.

## BACKGROUND

The human immune deficiency virus (HIV), a cytopathic retrovirus, is implicated to be the causative agent of the acquired immune deficiency syndrome (AIDS), by seroepidemiology, virus isolation, and molecular epidemiology (1,2). Infection with HIV-1 in vivo is usually associated with an asymptomatic interval which frequently lasts from several weeks to several years followed by a progressive degeneration of the immune and central nervous systems (3-6). HIV-1 virus selectively infects and kills OKT4+ helper/inducer lymphocytes which play a critical role in the regulation of the immune response (7). Viral replication involves the formation of an intermediate "provirus" stage in which a double stranded copy of the viral genome integrates into the host chromosome. This provirus can then remain in an inactive, quiescent stage or serve as the source of active viral replication following activation. The selective depletion of the OKT4 cells in the peripheral blood upon activation of the viral genome apparently leads to the observed

immunodeficiency in the latter stages of the disease, such that the infected individual becomes susceptible to opportunistic infections and dies.

Extensive immunologic, genetic and molecular analysis of the HIV-1 retrovirus in the last few years has shown the viral genome to be far more complex than that of most known retroviruses. The HIV-1 genome consists of a 9.7 kilobase RNA molecule in which nine genes have been identified to date. In addition to the structural genes for the core proteins (gag), the viral enzymes (pol), and the envelope glycoproteins (env), several accessory genes have been identified either serologically or as open reading frames in the viral DNA (8, Table 1). These accessory genes include the regulatory proteins tat, rev and nef, the viral infectivity factor vif, and two proteins of lesser defined function vpu and vpr (9-13). Sequential expression of the regulatory genes induces modulation of viral expression which appears to be the key event in determining viral replication and latency. Two gene products (tat and rev) have been shown to directly modulate viral expression via transcriptional (tat) and post-transcriptional (tat and rev) mechanisms (10). The transactivator gene (tat) is critical for virus replication and enhances transcription of genes linked to the long terminal repeat (LTR) (11). The rev gene (formerly known as art and trs) positively regulates the expression of structural proteins but negatively regulates the regulatory proteins including itself (12). In turn, the nef (formerly called 3' orf) gene product is a negative regulator of viral expression (13). The vif (formerly known as sor) gene product is essential for the efficient propagation of extracellular virus in target human cells in vitro. The function of the vpr gene product has not yet been well characterized, but it appears to be non-essential for viral replication in vitro. Vpu encodes a 16 kd protein and its expression, like vpr, does not appear to be required for viral replication (9). The interaction of these virus products with cellular host factors is a very complex one. A better understanding of these interactions will help facilitate the development of vaccines and therapies to combat HIV-1 infections.

The molecular basis of HIV-1 gene expression can be studied in an in vitro model system for AIDS, in which the virus can be propagated in PHA stimulated peripheral blood lymphocytes (PBL's) or in a variety of lymphoid cell lines (14). Molecularly cloned proviral DNA can be

transfected into these cultured cells to generate infectious virions and cytopathic effects in vitro which are indistinguishable from virus obtained by cocultivation of permissive cells with the PBL's of AIDS patients (15). Such studies with a cloned viral DNA have provided direct evidence that a product of the HIV genome mediates cell killing. Genetic manipulation to alter defined portions of the HIV-1 DNA clone using standard recombinant DNA techniques have permitted detailed studies of the viral determinants of pathogenicity, transmission, and other viral functions, and these approaches have been used in the present study.

Expression of the HIV-1 genome is tightly regulated by the activity of several viral genes and regulatory sequences. The tat gene located between the vif and env genes has been reported to enhance transcription of genes linked to the long terminal repeats LTR's in infected cells (tat response), and presumably plays a central role in HIV-1 gene activation (11). The gene is expressed as a 1.9-2.0 kb mRNA generated by two splicing events and has many similarities to the corresponding tat gene in HTLV-I and HTLV-II (20). However, whereas the tat I and II proteins are reported to enhance transcription, the tat III gene product was shown to enhance expression of protein at a post transcriptional level (21). Another gene designated art or trs, possibly expressed from the same mRNA which encodes the tat gene, also regulates HIV-1 virus expression post-transcriptionally, most likely by regulating the accumulation of genomic and spliced viral mRNA (12).

The level of gene expression has been reported to be dependent on functional elements within the LTR sequence of the virus, including; (i) a negative regulatory element (NRE), (ii) an enhancer upstream of the promoter or TATA box, and (iii) tat response region downstream of the promoter at -17 to +80 (22). The tat response region allows greatly increased expression of linked genes in HIV-1 infected cells relative to uninfected cells even if placed in a similar position downstream of heterologous promoters (22). The HIV-1-LTR was also reported to contain three Spl binding sites as demonstrated by point mutagenesis and protection experiments (23). However, the functional discrimination of these sites was only partially dissected. The HIV-1 LTR contains both cis and trans regulating elements which have not been precisely localized. The work described in this

report was designed to define the function of the regulatory elements and their interactions with the viral genome.

Recently, additional retroviruses, termed HIV-2, related to HIV-1 but more closely to some strains of simian immunodeficiency virus (SIV) (e.g. $SIV_{mac}$) were isolated from sick and healthy individuals (24-26). Seroepidemiological and laboratory studies suggest that although some HIV-2 isolates are associated with AIDS, others may be far less pathogenic (24,27). Thus, the HIV family may comprise a spectrum of human retroviruses with varying degrees of pathogenicity. The pathogenic potential of each HIV may in part be determined by its genetic structure. Thus, it may be instructive to compare the functional capacities of the regulatory genes and elements of HIVs possessing varying pathogenic potential.

Closely related to HIV-2 is the Simian Immunodeficiency Virus (SIV) which has been isolated from captive rhesus macaques with clinical signs of immunodeficiency (28). Interesting parallels can be drawn between the retroviruses HTLV-I, HIV-1, HIV-2 and SIV. Among these are: *(1)* a common major target cell (CD4+ T-lymphocyte), *(2)* association with diseases of the immune and central nervous systems, *(3)* presence of highly related viruses that naturally infect Old World primates, and *(4)* common regulatory pathways for viral gene expression. Experimental inoculation of SIV in macaques induces immunodeficiency at high frequency, providing an excellent animal model to study pathogenesis, prevention and treatment of human AIDS. Although macaques in the wild are not known to be infected with SIV, several other Old World monkey species (pig tailed macaques, sooty mangabey, macaque menestrina, African green monkeys) appear to be naturally infected with viruses related to $SIV_{mac}$ which is quite divergent from these other simian viruses. $SIV_{mac}$, however, is closely related to HIV-2, whereas $SIV_{agm}$ is equally distant from both HIV-1 and HIV-2 (29). The biological similarity among these primate viruses suggests evolutionary conservation of their functionally active genes.

In the course of this contract, full length clones of the HIV-2 and SIV genomes were prepared, and their DNA sequence was determined and analyzed. The HIV-2 and SIV genomes characterized to date were found to contain open reading frames corresponding to most of the

HIV-1 genes, although some differences were observed (30). An additional gene (vpx) was found in HIV-2, $SIV_{mac}$ and $SIV_{agm}$, and does not have an obvious counterpart in HIV-1. On the other hand, the vpu gene is found only in HIV-1. $SIV_{agm}$ appears to lack the vpr gene which is found in HIV-1, HIV-2 and $SIV_{mac}$. Conservation of the amino acid sequence in the putative functional domains of common genes and the biological similarities among these AIDS associated retroviruses would lead one to assume that what applies to HIV-1 should also apply to HIV-2 and SIV. A low level of conservation of the amino acids is observed among the regulatory proteins of $SIV_{mac}$, $SIV_{agm}$ and the various isolates of HIV-2 (rod, NIH-Z, SBL6669) and HIV-1. On the other hand, a high level of conservation is observed in the amino acid sequence of the gag and pol proteins. A comparison of all regulatory genes indicates that there are some short regions in regulatory genes which are conserved and are involved in maintaining the structure of the protein. The cysteine/arginine rich region in the first exon of the tat gene and the arginine rich region in the second exon of the rev gene are highly conserved among all these viral isolates (29).

The envelope gene of HIV-1 has been shown to be highly variable among different HIV isolates. Interstrain variations occur consistently within the same areas of the envelope gene. These same regions are also poorly conserved among the HIV-2 and SIV isolates characterized thus far. The HIV-1 transmembrane protein is 41 kD in size, while the SIV and HIV-2 transmembrane proteins are only 32 kD. This difference in protein size is apparently due to the presence of a translation termination codon in the env genes of SIV and HIV-2. Interestingly, the termination codon, is located in the same place in both viruses and is present in most of the SIV strains characterized so far and in an HIV-2 provirus. Several researchers have determined that this premature termination codon was the result of the manner in which the virus was propagated (31). Virus isolated from Hut78 cells, contained the premature termination codon, while virus grown in fresh lymphocytes exhibited full length envelope protein. Results such as these indicate that a better understanding of virus propagation and effects of mutation is required in order to fully understand the pathogenicity of the virus. Furthermore, the high level of homology that exists

between the human HIV-2 isolate and the simian SIV isolate suggests that HIV-2 infection of primates may provide a suitable model for AIDS.

## PROGRESS

### A. CHARACTERIZATION OF DELETION MUTANTS OF HIV-1

### 1. Rationale

To study the molecular basis of infectivity, cytopathogenicity, and formation of infectious particles, a series of mutant clones with changes in discrete portions of the HIV-1 genome was constructed by site-directed mutagenesis or by exonuclease cleavage and religation. The mutants used for these studies were based on the molecular clone pHXB2Dgpt, which contains the full-length, biologically active HIV-1 genome (9.6kb) flanked by the long terminal repeat sequences in the plasmid vector pSP62gpt (32). This construct can be propagated in either E. coli cells to generate large quantities of the plasmid or in a monkey cell line, COS-1 where all the viral genes can be expressed to produce fully biologically active virus capable of propagation in permissive host cells (PBLs, or CD4+ containing lymphocyte cell lines).

Since preliminary studies had implicated an involvement of the vif and nef regions of the HIV-1 genome in pathogenesis and transmission, these regions were selected for more intensive examination in the course of this contract, using a mutagenesis/biological activity assay approach. Another aspect of viral infectivity selected for closer study is the process of virion assembly, more specifically, the signal for packaging of viral genomic RNA into particles. Accordingly, viral mutants were prepared with defined alterations in three areas of the viral genome: the vif region, the nef and carboxy region of gp41, and the 5' packaging region. Deletion mutations in these regions were generated, methods for examining the various parameters of viral function in vitro were worked out, and studies on the correlation of viral gene function to specific loci on the viral genome were carried out.

Previous studies with mutants in the vif (sor) region indicated that this gene is conserved among all HIV-1 isolates as well as in SIV and HIV-2 (30), suggesting that this gene may be functionally significant. Our initial studies with mutations in this region have shown that vif (sor)

is required for efficient virus transmission in vitro, and these studies were further expanded in the course of this contract (18). Because of its role in enhancing infection, the gene was subsequently renamed viral infectivity factor (vif). The carboxyterminus of the gp41 glycoprotein which overlaps the nef region has been implicated in playing an important role in virus transmission and cell killing. The speculation that the carboxy terminus of the gp41 glycoprotein might have a direct role in T-cell killing by HIV-1 had come from a study in which a variant X10-1, derived from the pHXB2D with the deletion spanning the env and nef genes, was found to replicate but not to kill normal T cells (13). We hypothesized that the C-terminus of the gp41 transmembrane protein is vital to viral transmissibility and cytopathicity. We tested this hypothesis by using a panel of mutants which have deletions in the env, the nef, and/or 3' LTR to study the effects of the defined mutations on the replication, transmission and cytopathic potential of the virus in the human T-cell lines, H9 and Molt-3. Additional studies were designed to quantitate differences in the cell killing potential of the C-env mutant viruses. The cell lines ATH8 and SupT1 have been shown to be particularly susceptible to rapid killing by HIV-1, even at relatively low doses (10), and were therefore used in these studies, along with the normal host cell H9.

One approach for the production of defective HIV viral particles, which can be used as interfering particles for therapy or a source of vaccine, is the production of "empty particles" which do not contain viral RNA, and are therefore incapable of replication. Although much is known about HIV replication and the processing of structural components (gag, pol, and env) in infected cells, the precise mechanism by which genomic viral RNA is preferentially packaged into virion particles is unclear. Such a mechanism, presumably involves a recognition sequence in the viral genome which permits the viral assembly apparatus to specifically select and incorporate its own genomic RNA out of a vast array of viral and cellular mRNAs, and ribosomal and transfer RNAs found in infected cells. Studies in the avian and murine retrovirus systems have suggested that virus particle formation can occur in the absence of genomic RNA and that the sequences which fall between the 5' LTR and gag are critical for virus specific packaging (17-19). To determine whether analogous sequences in HIV are important in selective packaging of genomic RNA, we

produced a series of mutants of the biologically active molecular clones pHXB2-D and X10-1 and studied their biological properties. Our studies with mutations in the region between the 5'-LTR and gag, showed that these mutants give rise to morphologically normal viral particles, but transmit poorly via the cell-free mechanism to permissive cells. These mutant viruses were then propagated in culture and the ratios of RNA to antigen in the virions was carefully measured to demonstrate that these mutants are indeed defective in the packaging of RNA.

## 2. Experimental Methods

Construction of Mutants in vif

A series of mutant clones with changes in discrete portions of the HIV-1 genome were constructed by site directed mutagenesis or by exonuclease cleavage and religation of pHXB2Dgpt (Figure 1). The molecular clone pHXB2Dgpt contains a full length, biologically active HIV-1 genome (9.6 kb) flanked by the long terminal repeat sequences in the plasmid vector pSP62gpt which contains the xanthine guanine phosphoribosyl phosphatase gene (32). The mutant ΔS was prepared directly from pHXB2gpt by removal of the sequences between the NdeI and NcoI sites (nucleotides 4707 and 5259) and religation. Additional mutants were prepared by site specific mutations using oligonucleotide-directed mutagenesis. These mutations introduced translational stop codons at various points in the vif frame downstream of the vif/pol overlap (Figure 2). To construct these mutants the EcoRI to EcoRI fragment (nucleotides 4230 to 5322) of the HIV-1 genome of λ BH10 was subcloned into an M13 phage vector, and mutagenesis performed as described by Ivanoff et al. (33). Sequences in this region of BH10 differ from those of HXB2 only at nucleotide position 4506 which results in a proline to serine substitution in pol. Mutation 6.9 was introduced using the 25-mer (GGATGAGGGCTTTCTTAGTGATGCT), converting the tyrosine codon (TAT) at residue 55, into a stop codon (TAA). A similar approach was used to replace the serine codon (TCA) at position 42 with a stop codon (TAA) in clone 3.3 , and to change the glutamine codon (GAA) at residue 100 to a stop codon (TAA) in clone 153. Following confirmation by DNA sequencing the mutated fragments were subcloned into the original proviral clone.

Transfections and Cocultivation

A half-million COS-1 (simian fibroblast) cells were seeded into 25 $cm^2$ flasks one day prior to transfection. Transfections of 5 µg DNA from mutant plasmids, pHXB2Dgtp (positive control), or PSV2neo (negative control) were performed using the $CaCl_2$ precipitation technique and a 10% DMSO shock (15). The COS-1 cells were incubated in high-glucose Dulbecco's minimal essential medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. After 48 hours, 2 x $10^6$ polybrene treated (2 µg/ml, 37°C, 30 minutes) H9 or Molt-3 cells were added on top of the transfected COS-1 cell layer in a total of 4 ml media (cell to cell transmission). The coculture was maintained for 48 hours at which time the H9 and Molt-3 cells were separated from the COS-1 cells. These H9 and Molt-3 cells were then maintained in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS).

Viral Gene Expression by Deletion Mutants

The activity of reverse transcriptase (RT) of the spent medium from the transfected COS-1 cells was assayed from 2 days to 2 weeks after the transfection by the incorporation of $^3H$ dTTP into TCA precipitable material. Samples with a ratio of the incorporation using RNA vs DNA templates of greater than 3:1 were scored as positive. The production of the gag p24 protein, monitored weekly by immunofluorescent assay of cells fixed in methanol/acetone, was used to demonstrate viral transmission from the transfected COS-1 to H9 or Molt-3 cells.

In vitro Neutralization Assays

Neutralization activity of the various sera was determined as follows. The antibodies or sera are first heat inactivated at 56°C for 30 minutes and filtered through a 0.45 µm filter. For neutralizing activity assays, H9 cells are exposed to 2 µg/ml polybrene for 30 minutes, pelleted, and incubated with HIV-1 virus (1000 virions per cell) for 90 minutes. The cells are pelleted and diluted in RPMI containing 10% heat-inactivated fetal calf serum in the presence of serial dilutions of the test sera. Ten thousand cells are seeded in 200 µl per well of a 96-well flat bottomed tissue culture plate, and the plates are incubated at 37°C for 7 days. The cells are then examined for the

presence of HIV by immunofluorescence with an anti-HIV p24 monoclonal antibody. The neutralizing antibody titer is expressed as the reciprocal of the serum dilution at which the HIV-1 infection of H9 cells is inhibited by 50% relative to that obtained with control HIV-1 negative sera.

Construction of Mutants in the Carboxy-Terminal of gp41

The molecular clone pHXB2gpt was linearized with XhoI and digested bidirectionally with Bal31 nuclease and religated. In constructing clones X429 and X468, XbaI linkers with termination codons in all 3 reading frames (SMURF I linkers, Pharmacia) were inserted prior to religation. The mutations resulted in truncation of gp41 in some cases, and frame shifts due to the deletions leading to the addition of new amino acids in others. The expected amino acid sequences of all the mutants used in these studies was derived from the DNA sequence analysis of these mutants and is shown in Figure 3.

Cell-Free Transmission of HIV Mutants

Cos-1 cells were transfected with 5 μg of DNA from each of the mutants, pHXB2D or pSV2neo. After 48 hours incubation, the resulting virus was collected from the supernatant, and clarified of cellular material by centrifugation at 6,000 rpm for 20 minutes. The virus from the clarified supernatant was collected by overnight precipitation with PEG. Serial dilutions of this virus were used to infect H9 and Molt-3 cells. Cells were assayed at weekly intervals for the expression of HIV-p24 by reactivity with a monoclonal antibody to this antigen using immunofluorescence assays to obtain an inverse geometric mean titer (GMT).

Cytopathogenicity of Cell-Free Virus Preparations of Deletion Mutants

Polybrene-treated H9 cells, 3 x $10^6$ in 3 ml RPMI medium containing 20% heat inactivated fetal calf serum, were incubated with 300 x TCID50 (Tissue Culture Infectious Dose 50% = dilution of virus at which 50% of cells show cpe) of the respective virus preparations (or media as the control) for 1 hour at 37 °C. The cells (in 100 μl medium) were transferred to triplicate wells in 24-well microtiter plates, and 2 ml medium was then added to each well. Plates were fed with 1 ml

fresh medium weekly. Cell viability was assessed in 1 and 2 weeks of the incubation by exclusion dye 2% trypan blue.

Construction of Packaging Mutants

Packaging defective mutants were constructed by producing deletions in the region between the 5'LTR and gag coding sequences of the plasmid clone pHXB2gpt. This plasmid contains a unique BssHII site in the sequences which intervene the 5'LTR and the beginning of the gag gene. A series of deletion mutants about the BssHII site were generated using the Bal31 digestion-religation approach. Clones #3 and #11 are deleted 51 and 57 nucleotides, respectively (positions 225-276, and 225-282) upstream from the splice donor site (position 287). Clones #79 and Δ293 are deleted 8 nucleotides (position 311-319) and 35 nucleotides (position 293-328) downstream of the splice donor, prior to the start of gag. The parental plasmid from which the #3 and #11 mutants were derived, pHXB2gpt contains the intact, wild type genome of HIV-1. The mutant #79 was derived from X10-1gpt and Δ293 was derived from pHXB2 neo. The map locations of these deletions is shown in Figure 4. In all cases, the splice donor site (position 287) is preserved.

Transfection of Packaging Mutant DNA and Virus Isolation

The DNA from plasmids #3, #11, #79, Δ293 and pHXB2gpt were transfected into COS-1 cells following the calcium phosphate coprecipitation procedure (15). Each 100 mm plate contained 1 x $10^6$ COS-1 cells and was transfected with 10 μg of DNA. Following transfection, the cells were incubated in DMEM-10% Fetal Bovine Serum and 0.1% gentamicin. At 2, 4, and 6 days post transfection, the supernatant medium was collected and replaced with fresh medium. The spent supernatant (4 plates each) was clarified by centrifugation at 3,000 rpm for 20 minutes, and virus particles were collected by PEG precipitation.

Virus Isolation from Cells Infected with Packaging Mutants

Virus produced in COS-1 transfected cells was transferred to H9 polybrene treated cells by co-cultivation, and stably infected H9 cells were allowed to grow out. For virus isolations, the supernatants of infected cells were collected one day after feeding with fresh medium (RPMI-1640

with 10% Fetal Bovine Serum). For each mutant, 36 ml of supernatants (from about 0.6 x $10^6$ cells/ml) were clarified of cellular debris by centrifugation at 3,000 rpm for 20 minutes. The virus particles were then pelleted out of the clarified supernatant by centrifugation at 25,000 rpm for 1.5 hours in an SW28 rotor. The viral pellets were resuspended in 400 µl of PBS. Of this, 200 µl was extracted with phenol to isolate the RNA, and 100 µl was used for antigen capture assay.

Quantitation of Viral RNA and Viral Antigen

For the phenol extraction of viral RNA from virion pellets, 50 µg of E. coli tRNA was added as carrier, and the solution was digested with 0.1% SDS, 200 µg/ml Proteinase K for 30 minutes at 56°C. It was then extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), once with chloroform, and precipitated with two volumes of ethanol. Positive controls included the plasmid pBH10 and viral particles from H9 cells infected with HIV and treated in a similar manner to the mutant viruses. Serial dilutions of the precipitated RNA were denatured with formaldehyde at 65°C for 10 minutes, cooled on ice for 10 minutes, and slot blotted on BA85 nitrocellulose filters using the Minifold Apparatus from Schleicher and Schuell. Following hybridization with $^{32}$P-labeled pBH10 probes, the filters were autoradiographed. The amount of RNA present in each slot was quantitated by cutting the region of the slot out of the nitrocellulose filter and counting in a scintillation counter to determine the cpm hybridized. The amount of p24 antigen present in each sample was determined by the p24 antigen capture assay (Biotech Research Labs) using serial dilutions of antigens which gave readings in the linear range. Dilutions of pelleted virions from H9 cells infected with HIV served as positive controls. The amount of antigen was expressed as pg based on a standard curve with dilutions of known amounts of virus.

## 3. Results

a) BIOLOGICAL ACTIVITY OF vif MUTANTS

A series of mutations with premature stop codons in vif were generated and the biological consequences of these mutations were examined in functional assays. DNA from each of the vif deletion mutants was transfected into permissive lymphoid cells (H9, Molt4 and PHA-stimulated

blood mononuclear leukocytes), and the transfected cultures were monitored at approximately weekly intervals for HIV-1 expression. Molt3 cells, normal T cells, or H9 cells transfected with the deletion vif mutants consistently failed to express virus as detected by immunofluorescence, reverse transcriptase assays, or electron microscopy. Southern blotting analyses of these samples showed transient uptake of plasmid DNA but no detectable proviral sequences in long-term cultures. In contrast, cells transfected with pHXB2gpt reproducibly and rapidly yielded virus producing cells.

The ability of the vif mutants to produce virions was also examined by performing a series of transfections using the SV40 transformed cell line Cos-1 as a target. These experiments were aimed to amplify virus production by exploiting the capacity of the Cos-1 cells to promote episomal replication of plasmids carrying the SV40 origin of replication (including plasmids derived from pHXB2gpt) in transient assays. The transfected Cos-1 cells were then cocultivated with Molt3 cells which are permissive for HIV-1 infection. The supernatants from these cultures were concentrated 100-fold and examined for virus gene expression by Reverse Transcriptase assays, the formation of viral particles by electron microscopy, and for their ability to infect polybrene treated H9 cells. The transactivation activity of the mutants was determined by co-transfecting the mutant DNA along with pC15CAT into Cos-1 or H9 cells and measuring the resulting CAT activity (also see Figure 15 and Table 13).

As shown in Table 2, virus particles morphologically similar to wild type were recovered from Cos-1 cell cultures transfected with vif mutants 6.9, 3.3, 153, and ΔS. The level of virus production (both extracellular and budding virions) and transactivation potential was indistinguishable from that seen in pHXB2gpt transfected cultures. However, supernatants removed from vif mutant cultures (containing cell free virus) failed to infect H9 cells in repeated attempts and was transmitted poorly by coculture. In contrast, virus derived from clones with an intact vif frame was readily propagated by either approach. Normal amounts of gag, env and pol derived proteins were produced by all four mutant genomes; and assays performed in both lymphoid and non-lymphoid cells indicated that their transactivating capacity was intact and

comparable with wild type (34). This data shows that the vif gene, although not absolutely required in HIV virion formation, influences virus transmission in vitro and is crucial in the efficient generation of infectious virus.

Neutralization of Cell-to-Cell Transmission by vif Mutants

The above studies had shown that mutations in vif (sor) are not required for HIV virion formation, but influence viral transmission in vitro. The mechanism by which these mutants affect cell-to-cell transmission was further evaluated. Using soft-agar cloning techniques, we have isolated a number of cell lines expressing different vif mutant viruses. Previous studies have shown cell free transmission of these mutants to be defective (34). Since some of these lines are up to 95% positive for gag expression, they are very useful as inocula for neutralization studies for fresh polybrene treated Molt-3 cells. A standardized system using fixed numbers of Mitomycin-C treated Molt-3 cells infected with the various vif deletion mutant clones (ΔS, 3.3, 6.9, 153) was developed as an inoculum for fresh polybrene treated uninfected cells. This system shows predictable replication characteristics which allows investigation of the molecular basis of cell-associated transmission of HIV, by determining the effects of antibodies directed against specific cellular and viral structures. For example, antibodies such as OKT4A, OKT4, and Leu3a against CD4, Leu10 against HLA-DQ, Anti-HLA Class II antibodies, anti-gp120, gp41 and p24 monoclonals, and selected infected patient sera can be used to examine the role of various antigens in cell-to-cell transmission. Shown in Table 3 are typical kinetics for this assay. Up to 30-50% of the cells express HIV-p24 at 7 days post infection, and infected cells continue to persist in the culture for up to 28 days. Various vif mutant infected cells lines have been cloned in order to obtain more homogeneous populations to use as inocula.

In an attempt to dissect the molecular basis of cell-free or cell-associated transmission , we examined the ability of a variety of human HIV sera to neutralize the transmission of the mutant viruses to H9 cells. For this assay, H9 cells were infected with 4 times the infectious dose (4 x TCID50) of HXB2D or HTLVIIIB (wild type virus) in the presence of various dilutions of sera. Cell-associated transmission was determined in a similar manner, except that the cell lines

expressing the vif mutant viruses were used as the source of the infecting virus. Expression of virus was monitored at 2 weeks post infection by immunofluorescence with anti HIV-1 p24. The serum dilution which gave a 50% reduction in titer was determined. OKT4A, but not OKT4 is extremely efficient in blocking cell-associated transmission, implying that the gp120-CD4 interaction is still required for cell-to-cell spread of the mutant viruses. The results with the human sera are shown in Table 4. Some sera (HS06 and HS12) neutralize cell-free transmission but not cell-associated transmission of the mutant virus. Other sera (HS02 and HS22) neutralize cell associated, but not cell-free transmission. These results suggest that different epitopes may be involved in the cell-cell versus cell-free transmission of HIV.

Mode of Viral Transmission as a Function of Progression to AIDS

Since different epitopes in vif have been implicated in the transmission of HIV-1 by both the cell-free and cell-associated route, we asked whether the mutants in vif could be used to determine whether the mode of virus transmission changes in infected individuals in the course of progression to AIDS. The wild-type virus (HXB2D and HTLV-IIIB) have been shown to transmit primarily via a cell-free route in culture, whereas mutants in vif transmit primarily via the cell-associated route. Since sera from different individuals can be shown to preferentially neutralize virus transmission by the cell-free or cell-associated route, we reasoned that neutralization experiments on these viral clones using sera from individuals at different stages of progression could provide us with insight as to the relationship, if any, between progression to disease and the preferred mode of transmission.

Two sets of sera, one from a patient who progressed to AIDS and one from a matched patient who did not, were examined for their ability to neutralize transmission of wild type virus HXB2D or HTLV-IIIB by the cell-free route. Neutralization of cell-to-cell transmission was examined with clonal cell lines infected with mutant viruses AC4/AEC1 and DA4/Xgpt with deletions in vif. Inverse geometric mean titers (GMT) of the different virus preparations using inocula four times the 50% infectious dose (4 x TCID 50) were determined. Titrations and neutralization assays were done with an immunofluorescence technique to detect HIV-1 p24 expression. The dilution of

serum which gave a 50% reduction of virus titer was the end point used. The results of these studies are shown in Table 5.

No significant differences in neutralization of cell-to-cell transmission (AC4/AECl and DA4/Xgpt) by the sera from the patient who progressed to AIDS was observed relative to the one who did not progress. Both sets of sera were not very effective in blocking this mode of transmission. Somewhat better neutralization was observed for the cell-free transmission route (HXB2D and HTLV-IIIB), with the serum from the patient who had progressed to AIDS giving an appreciably higher titer.

A similar study was performed to determine whether the method of transmission of HIV-1 correlated with the stage of the disease. In this set of experiments, the ability of sera from a patient at WR Stage 2 to neutralize virus transmission by either of these two mechanisms was compared to the sera from the same patient at WR Stage 6. The results, shown in Table 6, indicate that although all neutralization titers were relatively low, some differences were apparent. The sera at the early stage of progression was more effective in neutralizing virus transmission by the cell-free route. At late stage disease, however, the neutralization titer of cell-free transmission had diminished appreciably. The ability of this sera to neutralize the cell-to-cell transmission route, had on the other hand, increased by over 8-fold. These observations suggest that changes in immune activity in late stages of AIDS are more pronounced in neutralizing cell-free than cell-to-cell spread of virus.

b) STUDIES WITH C-TERMINAL DELETION MUTANTS

Cell-to-Cell Transmission Properties of Deletion Mutants in the Carboxy Region of env

Studies were designed to examine the biological activity of the nef gene, which also overlaps the carboxy region of env and the 3' LTR. Mutants with deletions at the 3' end of the env, nef, and 3' LTR were transfected into cells of the monkey fibroblast cell line Cos-1. Replication was determined by expression of viral reverse transcriptase activity in the cell free supernatants of transfected Cos-1 cells, and by the appearance of HIV-1 p24 in the cells in immunofluorescence assays. The ability of transfected cells to transmit infectious virus to uninfected cells was also

examined by culturing transfected Cos-1 cells with polybrene treated, H9 or Molt-3 cells for two days. The H9 and Molt-3 cells were then separated from the Cos-1 cells and incubated separately.

Shown in Table 7 is a summary chart reflecting the kinetics of the appearance of Reverse Transcriptase (RT) activity and p24 gag protein expression in H9 and Molt-3 cells. Mutant clones with deletions in the carboxy region of gp41 were observed to be capable of producing reverse transcriptase even when the mutations overlapped into the nef and LTR regions. Mutants X360 (87-amino acid deletion in the gp41) and X327 (177-amino acid deletion in the gp41) could not produce this enzyme since their deletions affected the tat/rev genes which are essential for the production of infectious particles. All the other mutants examined could propagate in H9 cells to establish infection in 4 weeks, although two mutants X295 (14-amino acid deletion in gp41) and X329 (no deletion in gp 41) propagated slowly at first. The most disparate transmission of mutants in H9 versus Molt-3 cells was observed in mutants X429 (15-amino acid deletion in the gp41), X269 (17-amino acid deletion in gp41), X468 (33-amino acid deletion in gp41), and X362 (37-amino acid deletion in gp41) which transmitted relatively efficiently to the H9 cells but transmitted very poorly, if at all to Molt-3 cells.

Quantitation of Cell-Free Transmission of Mutant Clones in H9 and Molt-3 Cells

To measure the relative differences in the ability of the mutant clones to transmit in a cell-free manner, Cos-1 cells were transfected with the mutant DNA's and the resulting viral particles released to the culture fluid were collected from clarified medium by PEG precipitation. Serial dilutions of this cell-free virus preparation were then assayed on H9 and Molt-3 cells. The dilution which produced a cytopathic effect in 50% of cultures ($TCID_{50}$) was determined. The cells were also examined by immunofluorescence with monoclonal antibody to HIV p24 (BT-3) and the multiplicity of infection (MOI = number of virions per cell) required to produce a 50% reduction in the number of fluorescent cells was calculated. The number of viral particles in each preparation was determined by two independent methods. The physical viral particle count in each preparation was calculated on the basis of electron microscopy. In addition, the reverse transcriptase activity of these preparations was also measured.

The results of these studies are summarized in Table 8. These results indicate that while virus particle counts and RT activity of these preparations varied only about six-fold among the various clones examined, the $TCID_{50}$ values varied over two orders of magnitude, even when adjusted on a per-particle basis. There was no simple, direct correlation between infectivity and the length of the C-terminal deletion. Poorly infectious clones X468 (-33+0) and X429 (-15+4) required 910 virions per cell and 2700 virions/cell for infectivity, respectively, whereas the parental HXB2 required only 18 virions per cell for 50% infectivity at two weeks. Surprisingly, two clones with C-terminal modifications similar to those above, X362 (-37+18) and X269(-17+2), possessed cell-free infectivity indistinguishable from the parent (3 virions/cell and 22 virions/cell, respectively).

The results of our studies of RT production and transmission to H9 and Molt-3 cells of the C-env mutants are summarized in Table 9, which also shows the location of the deletion and the resultant amino acid changes. All mutants except clones X360 (-187) and X327 (-177), which have deletions extending into the tat/rev regions rendering them incapable of growth after transfection into Cos-1 cells, produced extracellular reverse transcriptase activity after transfection into COS-1 cells. Mutants with deletions of -0 to -4 propagated well in both H9 and Molt-3 cells, except for X358 (-6), which contained a deletion of the 3'-LTR and was incapable of replication in either cell line. Mutants X429, X269, X468, and X362, containing deletions of 15-37 C-terminal residues, propagated in H9 cells somewhat more slowly than the parental HXB2 clone and failed to establish productive infection in Molt-3 cells by coculture. Clones with deletions of over 40 amino acids (X274 and X194) also contained deletions in the 3'-LTR and were unable to replicate in either H9 or Molt-3 cells.

Cytopathogenicity of Mutants in the Carboxy Region of env

Additional studies with the C-env mutants were designed to examine their cytopathic properties. Viral particles of selected mutants at 300 times their TCID50 concentrations were used to infect H9 cells and cell viability was monitored weekly for a period of two weeks. The cell viability, used as the measurement of viral cytopathogenicity on H9 cells, showed that mutants

X10-1 (5-amino acid deletion), X295 (14-amino acid deletion) and X269 (17-amino acid deletion) had little or no cytopathic effect by two weeks (Figure 5). When the deletions extended to 33 amino acids (X468), cell viability decreased to 39%. As deletion further extended to 37 amino acids from the 3' end of the gp41 (X362) cell viability decreased to 32%. This reduction in viability approached that obtained with the parental viral clone pHXB2D (24% viability). It is interesting to observe that mutant X9-3 (5-amino acid deletion) had 41% cell viability.

The cytopathic properties of mutants in the carboxy region of env on H9 cells were also compared to their infectivity on the highly susceptible cell lines SupT1 and ATH8. The cytopathic effects of different clones as cell-free virions were examined using virus preparations adjusted for infectious dose (H9 and SupT1) or a fixed multiplicity of infection (ATH8). All mutants displayed reduced cytopathic effects on H9 cells compared to the HXB2 parent (Figure 5), though X10-1, X295, and X269 were least cytopathic. Using SupT1 cells, X362 and X269 displayed markedly reduced cytopathic effects, while X10-1, X9-3, X295, and X468 were more cytopathic, though still less so than HXB2. All mutants were cytopathic to ATH8 cells, though X10-1, and X9-3 were significantly less cytopathic than HXB2, even when adjusted for infectious dose. Replication of the different clones during these experiments was comparable, as judged by p24 positivity by immunofluorescent staining (35).

The cytopathic potential of the different C-terminal deletion mutants was also examined using a new technique involving coculture of infected cells with exquisitely susceptible target cells. The cell lines ATH8 and HPB-ALL have been shown to be particularly susceptible to rapid killing by HIV, even at relatively low doses of virus. The ability of mutant virus produced in H9 or Molt-3 cells to kill these indicator cell lines by a cell-associated mechanism was examined. Unexpectedly, mutants previously described as non-cytopathic (X10-1 and X9-3) as cell-free virions appeared to show marked cytopathic effects when cocultured with the indicator cells ATH8 and HPB-ALL (Table 10). Mutant X362 which transmitted poorly to Molt-3 cells, nevertheless was still capable of killing the indicator cells particularly the ATH8 cells. The indicator cell lines were more susceptible to killing by X10-1, X295 and X362 when they were propagated in H9 cells than

when the same mutants were propagated in Molt-3 cells. These observations suggest that cellular factors also contribute to the cell killing property of HIV. These cellular factors interact with different viral determinants in bringing about the cytopathic effect.

c) STUDIES WITH PACKAGING MUTANTS

Characterization of Packaging Defective Mutants

Several candidate packaging mutants were generated and examined for their ability to give rise to infectious (RNA containing) virus particles. Plasmid DNA from each clone was transfected into Cos-1, and the culture supernatants of transfected cells were analyzed for the presence of reverse transcriptase activity and the appearance of virus particles by electron microscopy. These supernatants were also examined for the presence of infectious particles by cocultivation with recipient H9 cells. The transmissibility of the mutant viruses was monitored by assessing the increase in HIV expressing cells in the coculture H9 population with time.

Clones #3, #11, #79, and Δ293, all gave rise to morphologically normal virus particles, as examined by electronmicroscopy, and were capable of eliciting Reverse Transcriptase activity upon transfection of Cos-1 cells. The transmissibility of viruses from clones #11, #3, and #79, however, was impaired with respect to wild type virus. These viruses were poorly infectious while virus derived from clone Δ293 appeared to be resistant to propagation (Figure 6). This data indicates, that although viral particles and virus specific enzymatic activity is produced at near wild type levels, the infectivity of these mutant viruses is impaired.

Viral RNA Production by Packaging Mutants

The transfected cells and the resultant viruses were examined for evidence of viral RNA production and packaging. Cos-1 cells were transfected with plasmid DNA from clones #11, Δ293, and pHXB2 (wild type virus). RNA was prepared from cellular lysates using the hot-phenol method, and analyzed by Northern blotting. Transcripts of sizes 9.5 kb, 4.5 kb, and 2.0 kb were evident in all transfected cells, indicating that viral RNA expression by these mutants was not compromised (data not shown).

Kinetics of Viral Antigen and RNA Production by Packaging Mutants

The packaging mutant DNAs were used to transfect COS-1 cells by the calcium phosphate co-precipitation method. The supernatants of transfected cells were monitored for the appearance of viral antigen and RNA at various times post transfection. The results of these analyses are shown in Figure 7. By 2 days post transfection, HIV-1 p24 antigen begins to accumulate in the supernatant of cells transfected with #3, #79 and gpt plasmids, and persists at 4 and 6 days. Virus specific RNA also appears at 2 days post transfection, but becomes somewhat reduced at day 4 and 6, possibly due to instability of the viral RNA when incubated at 37°C. These results suggest that virus is produced only transiently in the transfected cells.

Quantitation of Viral RNA and Antigen in Virus Produced by Transfected Cells

To quantitate the relative amount of viral antigen and RNA in viral particles produced by packaging mutants, the spent media of COS-1 transfected cells at 2 days post transfection (the time of maximum virus activity) was used as the source of viral particles for quantitation. The viral particles were precipitated from the clarified supernatants by PEG precipitation, and aliquots assayed for viral RNA and viral p24 antigen content. The amount of viral RNA produced per virus particle was standardized by dividing the cpm obtained by slot blot hybridization (RNA content) by the pg p24 antigen as determined from the optical density (OD) obtained from antigen capture analysis (antigen content) of the viral particles pelleted from the medium at two days post transfection. The results are shown in Table 11. The pelleted mutant viruses #3 and #79 contained approximately the equivalent amount of viral antigen as had been produced by cells transfected with the wild type HXB2-gpt plasmid, or by H9 cells infected with HIV-1. The amount of viral RNA, however, was significantly lower in the #3 and #79 viral particles, even when corrected by the quantity of antigen present. The untransfected COS-1 cells gave only background OD and cpm by both the antigen capture and slot blot assays. These results suggested that the mutations resulted in packaging defective viral particles which produced near normal levels of viral particles, but which were depleted of viral RNA and therefore exhibiting reduced infectivity.

Quantitation of Viral RNA and Antigen in Stably Infected Cells

The transfection studies made quantitation difficult, since the virus was only expressed transiently in the transfected cells, and only relatively low amounts of viral particles were actually produced. The transfected COS-1 cells were therefore co-cultivated with H9 cells and stocks of the mutant viruses were grown in H9 cells to provide a larger and more stable source of viral particles for study. The infected H9 cultures were continuously passaged and expanded over a period of several weeks and the percentage of virus infected cells was monitored by histocytochemical staining of cells with a monoclonal antibody to HIV-1 p24 and an alkaline phosphatase anti-alkaline phosphatase (APAAP) detection system. Stable lines of H9 infected cells were thus derived from COS-1 cells transfected with the mutant viruses #3, #79, and #82 as well as from those transfected with the DNA from pHXB-2gpt, the original plasmid containing the intact HIV-1 genome from which these mutants were generated. These lines contained more than 50% infected cells each as assayed by the APAAP procedure and the percentages of infected cells remained stable with time.In attempting to quantitate the amount of viral antigen present in virions, we noticed that PEG precipitation of cell-free supernatants gave variable results from experiment to experiment. This may have resulted from the variable extent of cell lysis during the cell culture or processing leading to the release of cellular debris into the supernatant. The presence of soluble p24 antigen in this debris can be variable, contributing to the inconsistencies in the amount of p24 observed following PEG precipitations. To circumvent these difficulties, the virions were pelleted out of the clarified culture medium by ultracentrifugation. These purified virions were then resuspended and used for the quantitation of viral antigen and RNA content. Serial dilutions of the virus pellet were assayed for p24 antigen by the antigen capture assay (Biotech). Duplicate samples were extracted, slot blotted onto nitrocellulose membranes and hybridized with $^{32}P$ labeled pBH10 probes. The amount of viral RNA in the sample was quantitated by cutting out the region of the slot and counting in a scintillation counter (Figure 8). The linear relationship between cpm and the amount of viral sequences using pBH10 as a target is shown in Figure 9, which demonstrates that this assay can be used for the quantitation of viral RNA content. The antigen

content of cell-free virions (antigen capture results expressed in pg virus) was plotted against the dilution factor, and the linear region of curve was used for the calculations (Figure 10). Similarly, the RNA content of the virions preparations were calculated by plotting cpm versus dilution and the linear region of the curves used (Figure 11). To calculate the total antigen and total cpm, the linear regions of the dilution curves were chosen, and the amount of antigen (pg) or RNA (cpm) was multiplied by the dilution factor to obtain the total amount of each present in the sample (Table 12). The ratios of RNA to antigen in the virions could then be calculated.

As can be seen from the ratios of RNA to antigen in the virions, virus obtained from transfections with the HXB-2 gpt DNA which contained the wild type HIV-1 genome gave very similar ratios to that obtained from wild type HIV virions grown in H9 cells. Mutant #3 and #79 gave significantly lower ratios of RNA to antigen (about 43% and 14%, respectively of wild type).

## 4. Discussion

Mutations in vif

The studies described in this report have shown that construction and analysis of deletion mutations is a very powerful approach to elucidating gene function. We have shown that removal or truncation of vif results in virus progeny that exhibit a greatly reduced (> 100-fold) infectivity. The expression of vif gene function is not crucial for the production of morphologically intact virus particles, but mutant viruses are defective in their ability to infect permissive cells by the cell-free route. Furthermore vif mutant viruses were also transmitted less well under co-culture conditions (in which cell to cell transmission is likely to be important). Interestingly, the effects of truncating vif (in the case of mutants 3.3, 6.9, and 153) were similar to its complete removal (in the case of mutant ΔS) suggesting that the carboxy-terminal portion of vif (downstream from residue 100) may include a functional domain. However, the possibility that vif is non-functional because it is deprived of critical elements in the carboxy-terminal of the protein (necessary for the correct folding or processing) is not excluded. Since the level of viral RNA, proteins and viral particles produced by vif defective genomes could not be distinguished from that of wild type, we suspect

that vif exerts its effects at a post-transcriptional and post-translational level which operate to regulate virus expression in infected cells.

While the mechanism of how vif enhances virus propagation is not yet understood, several distinct mechanisms could be postulated. It is possible that vif is a structural component of the virion particle which acts as a 'second envelope' required for efficient transmission. Since it is difficult to detect vif in as large amounts as gp120 and gp41 (in either infected cells or virions) additional studies are necessary to evaluate this likelihood. Alternatively, the vif gene might be involved in stabilizing or processing envelope so that assembly of infectious virus is increased, or in potentiating the cellular environment in which viral replication occurs.

Our studies with deletion mutations in vif, also demonstrated that different viral epitopes in the vif region are involved in the transmission of the virus by a cell-to-cell or a cell-free mechanism. Using a series of human anti-HIV-1 sera, we had found that some sera which neutralize cell-free transmission by mutant viruses did not neutralize transmission by direct cell-to-cell contact. Other sera gave the opposite result. These studies were further extended to determine whether the mode of vif-mediated transmission correlated with progression to AIDS. We asked whether antisera from patients who progressed to AIDS are more efficient in neutralizing transmission through the cell-free or the cell- to-cell route, and whether this neutralization differs from that in patients who do not progress to AIDS.

Preliminary studies relating the mode of virus transmission as a function of progression to AIDS, suggest that during late stages of disease, the neutralizing antibodies in serum are more effective in blocking cell-to-cell transmission than cell-free transmission by virus (Table 6). The reverse situation exists at early stages of disease. Thus changes in neutralizing activity appear to accompany progression to AIDS. Since the virus has spread to many more cells at late stages than at early stages, this data may suggest that virus is transmitted primarily by the cell-to-cell route in early disease, but cell-free transmission is more important at late stages.

It is not clear from our studies whether neutralization assays of this sort at early stages can be used to predict rapid or slow progression to AIDS. The individual who progressed to disease had

significantly higher neutralizing titers for the cell-free transmission route than did the individual who did not progress (Table 5). However, if the cell-to-cell transmission route is more significant in early disease, neither the slow nor rapid progressing individuals had significant neutralization titers for this route. These studies would have to be carried out on a larger sample population before any reliable conclusions could be drawn about the predictive value of such assays in progression. However, this approach may be useful to dissecting certain immunological and viral changes which accompany progression to AIDS.

Mutations in nef

Studies of the biological activity of mutations in the 3' env/nef region have shown that mutants of up to 37 amino acids deleted from the 3' end of the gp41 are still capable of producing reverse transcriptase upon transfection into Cos-1 cells and of transmitting virus to H9 cells. However, mutants with 15 or greater amino acid deletions cannot propagate in Molt-3 cells. This preferential transmission in H9 cells suggest that certain cellular factor(s) of Molt-3 and the area between the last 14 to 15 amino acids of the gp41 seem to work in an accord to modulate viral transmissibility and that cellular tropism might play a role in viral transmission. Transmission of mutants X429, X269, X362, X468 was markedly reduced in Molt-3 cells compared to H9 after coculture with transfected Cos-1 cells. Two of these four clones, X468 and X429 exhibited poor cell-free infectivity for Molt-3 cells, but still showed preferential transmission in H9 cells, indicating that the cell-free route alone was not sufficient to establish a successful infection. Therefore, the areas between the last 15 and 17, and between the last 33 and 37 amino acids of the gp41 appear to be important to cell-to-cell transmission. In addition, the cytopathic effects of mutant clones with C-terminal deletions of only 5 amino acids (mutants X10-1 and X9-3), have previously been shown to be reduced, and the present study demonstrates that X10-1 is much less cytopathic than X9-3.

These results definitively localize the critical region for cell-cell transmission to the 17 carboxy-most amino acids of gp41. Two functional areas of importance in this very small region can be postulated. An area important in cell killing lies in the last 6 amino acid residues, or perhaps

in only the last 3 amino acid residues since X10-1 differs only in the highly hydrophobic 'ILL' sequence in this region. The other area, important for cell-to-cell transmission lies in the last 17 amino-acid residues, but does not include deletion of the last cysteine residue in gp41. Although no obvious relationship between the length of the deleted C-terminal portion of gp41 and viral infectivity is apparent from these findings, the mutants generally exhibited mildly to moderately impaired cell-free infectivity on a per virion basis (on either H9 or Molt-3 cells). Furthermore, mutants with deletions of over six amino acids that possessed significant cell-free infectivity (X269, X295, and X362) had relatively higher titers on H9 compared with Molt-3 (4.8:1 versus 5.7:1), whereas the parental HXB2 and clones with smaller deletions (X10-1 and X9-3) had equivalent infectivity on both cell lines (1.2:1 versus 1.4:1). The differences in cell-free infectivity do not appear to be sufficient to account for the striking differences among the mutants in transmission by coculture and replication in the H9 versus Molt-3 cell lines (e.g., X269 could not be transmitted in initial coculture to Molt-3, but cell-free virus grown in H9 was infectious). These observations can be contrasted with those obtained with the vif deletion mutants, which are deficient in cell-free infectivity and have been shown to replicate efficiently in Molt-3 cells but were unable to replicate in H9 cells.

The use of the highly sensitive cell lines ATH8, SupT1, and HPB-ALL have permitted us to more closely examine the cell killing properties of the C-env mutant viruses. Studies with these cells have shown that even when transmission to Molt-3 cells can not be demonstrated by some of these mutants (X429 - X362), they are still capable of killing the indicator cell. The studies on the cytopathicity of the C-env mutants suggest that the region between the last 14 and 17 amino acids seems to be important to viral cytopathicity since the cytopathicity started to be reduced as deletions extended beyond this region. We speculate that deletion of 14 amino acids from the 3' end of the gp41 altered the conformation of this protein in such a way that the virus became less cytopathic. When the deletion went further to 33 amino acids and beyond, this effect was reversed due to another alteration of the conformation. Infectivity and cytopathicity (as well as syncytium production) were not necessarily coupled, as illustrated by clone X269, which displayed high

infectivity but was minimally cytopathic. However, cytopathic effects were found to vary considerably depending upon target cell line and type of inoculum (i.e., cell-free versus cell-associated). The studies with mutants in the carboxy region of env, therefore, demonstrate that this region plays an important role in the transmission properties of the virus as well as in cell killing. Both these processes, however, appear to be quite complex and involve other cellular factors, and perhaps additional viral factors which will require additional study.

Perhaps the most significant observation is that the cytopathic effect of the C-terminal gp41 deletion mutants in coculture may far exceed that of cell-free virions, and equal that of wild-type virus. While this is somewhat discouraging, it certainly indicates that any supposedly attenuated strain of HIV will need to be tested in this fashion (cell-associated cytopathogenicity). These studies therefore suggest that caution should be used in selecting candidate immunogens for vaccination derived from the HIV env region, since they might themselves be cytopathic for susceptible cells. Furthermore, novel therapeutic approaches such as those being developed to specifically block tat functions may be ineffective unless they are designed to completely prevent expression of HIV in infected cells.

Mutations in Packaging Region

The studies with the packaging mutants have demonstrated that the region between the 5' LTR and gag contains the signal for the packaging of the RNA into virions. Deletions in this region resulted in formation of viral particles which were morphologically normal but had reduced ability to transmit by a cell-free route. Two mutants, Δ293 and #79 have been identified as potential candidates for production of empty particles. These mutants synthesize all classes of viral mRNA, but the decrease in infectivity and viral RNA content of virions had suggested a possible defect in packaging. The quantitation of viral antigen and RNA present in cell-free virions demonstrated that these mutants produced viral particles containing normal levels of p24 antigen, but greatly reduced amounts of viral RNA. The decrease in RNA content presumably account for the lower transmissibility of these mutants by the cell-free route. It should be noted, however, that these particles are not totally devoid of viral RNA since small residual levels of viral sequences can

still be detected. Furthermore, these mutants are viable in that cells carrying the virus maintain the virus for many passages with no apparent decrease in viral antigen levels. The cell-free virus also contains very low levels of infectious particles, but less than 5% of that of wild type virus. The residual levels of viral RNA in these virion preparations preclude the use of these mutants as vaccine per se. However, combinations of this mutation along with other defects in replication, may permit the production of antigenically normal viral particles which are incapable of producing disease.

B. STUDIES OF HIV-1 TAT FUNCTION

## 1. Rationale

The transactivator gene, tat, is an extremely strong positive regulator of the expression of HIV genes, enhancing the production of all viral structural proteins, including itself (11). Since this gene may play an important role in reactivating latent virus, and therefore in the progression to disease, a clear understanding of the mechanism of tat activation is necessary to design regimens to keep the infection under control. The tat gene product has been shown to enhance transcription of genes linked to the long terminal repeat (LTR) of HIV. The studies described in this section of the contract were designed to elucidate the interaction of HIV-1 LTR, tat, and related factors in the regulation of viral gene activation.

A series of deletions within the HIV-1 LTR was introduced to localize the cis acting elements upstream of the promoter within U3 and to localize the trans acting response sequences in the R region. To study the resulting LTR function of these alterations, these constructs were linked to the chloramphenicol acetyl transferase gene (CAT) for assay by transfection into HIV-1 infected and uninfected cells. The location of cis acting regulatory elements could then be deduced from the analysis of deletion clones in LTR which fail to produce CAT expression. Trans acting regulatory elements can be identified by co-transfection of LTR-CAT with other plasmid constructs not linked to CAT but containing deletions in LTR, tat or art/trs. The regulation of genes linked to LTR, was also studied by examining other viral and cellular factors/proteins which play a role in HIV activation by tat. Our studies had suggested that tat functions at both the transcriptional level by promoting increased transcription of viral mRNA, as well as at the post-transcriptional level. The mechanism of postranscriptional activation by tat, was therefore also examined in an in vitro system which allowed us to study these mechanisms in more detail.

The enhancement of HIV transcription by tat proceeds by a complex mechanism that involves the interactions between tat protein, the binding elements in the LTR, and various other cellular or viral proteins or factors. Several reports have described the activation of HIV replication and

production of virus from latently infected cells by agents that induce lymphokine synthesis and secretion (37, 38). Jurkat cells have been used extensively in studies of the production of interleukins in response to various external stimuli. By treating Jurkat cells with a combination of phytohemagglutinin (PHA) and phorbol myristate acetate (PMA, 4-b-phorbol-12-b-myristate-na-acetate), or either agent individually, transient expression of a reporter gene linked to the HIV-LTR was shown to be elevated compared to similarly transfected Jurkat cells grown in normal media (39, 40). This response was amplified if the HIV trans-activator gene (tat-III) was cotransfected with the HIV-LTR reporter gene construct (12). Induced expression of the reporter gene was shown to coincide with the appearance in nuclear extracts of an activity that retards the migration of a DNA fragment containing the HIV enhancer sequence in a gel retardation assay (40). This activity was proposed to be identical to the NF-KB (41,42) factor that is detected in kappa-immunoglobulin light chain producing cell lines by the gel-retardation assay. In the present studies, the role of cellular proteins in the regulation of genes linked to the HIV-LTR, and the relationship between the LTR binding proteins in stimulated Jurkat cells to those found in H9 cell was examined.

Immune activation of infected T cells has been shown to stimulate HIV-1 replication (36,37,43,44) perhaps through effects on regulatory elements located within the long terminal repeat (LTR) of HIV-1 (40). The mechanism by which immune activation augments replication of HIV-1 in infected T cells was investigated by examining the effects of four different classes of T-cell mitogens on the HIV-1 long terminal repeat (LTR). The mitogens studied include phytohemagglutinin (PHA), a mitogenic lectin; phorbol 12-myristic 13-acetate (PMA), a tumor promoter; ionomycin, a calcium ionophore; and Tat -1, the transactivator protein from the human T-cell leukemia/lymphoma virus type 1 (HTLV-I) (45).

Studies of the interaction between tat and LTR have suggested early in the course of this contract that the RNA within the TAR region of the HIV-1 LTR is capable of forming a stable stem loop which may be critical to transactivation. Both transcriptional and post-transcriptional effects of Tat have been observed, further suggesting that the recognition of cis-acting elements in RNA

may be involved in Tat function. These observations suggested that Tat protein may bind directly to the RNA, either alone, or in combination with cellular factors. Experiments were therefore designed to test whether such binding can actually be demonstrated. The functional domains of Tat protein were examined by the generation of a series of site-directed mutations within the amino terminus of this protein. Sequence analysis of other eukaryotic transactivators (e.g. the yeast trans-activator GAL 4) has shown that many of them contain distinct DNA binding and activating domains (46). The activator regions of trans-acting factors share no known sequence homology other than the acidic and often amphipathic character that appears important for function. We therefore searched for a similar domain in HIV-1 Tat in order to identify potential activating domains. The amino-terminal region of HIV-1 Tat was found to constitute an acidic domain. Three acidic residues at positions 2, 5, and 9 are found within this region. The presence of acidic residues at these positions is consistent with the periodicity of an $\alpha$ helix, and would place all three acidic residues on the same face of an $\alpha$ helix if one were formed. Examination of the amino-terminal region of Tat from several isolates of HIV-1 showed that the sequence of these first 13 amino acids is highly conserved (47). To examine the function of acidic residues within the amino terminal of HIV-1 Tat, a series of mutations was generated in this region using site directed mutagenesis, and these sequences were inserted into the expression vector $pSV_L$ (under the control of the late SV40 promoter). The functional effects of these mutations on the trans-activation of the HIV-1 LTR was then correlated with the effects on charge and predicted structure.

## 2. Experimental Methods

Plasmid Clones

Plasmid pSVOCAT, pSV2CAT, and RSVCAT were obtained from Dr. Howard (48). Clone pC15CAT was constructed by blunt ending the PstI cDNA insert of clone pC15 (20) with T4 DNA polymerase and the four NTPs (Figure 12). HindIII linkers were added and the resulting fragment was cloned into the HindIII site of pSVO. DNA sequence analysis confirmed that the entire 3' region of the C15cDNA insert was present in C15CAT including the poly A tail. VHHCAT was

constructed by cloning the most 3' HindIII fragment of the HXB2 provirus (49) into the HindIII site of pSVO.

Construction of 3' Deletion Mutants

A series of deletion mutations in the HIV-1 LTR region was constructed from pC15CAT by digestion with Kpn I followed by digestion by Bal I exonuclease for various periods of time, and religation (Figure 12). The resulting clones were designated -278, -176, -117, -103, -65, and -48 according to the position of the sequence of HIV-1 LTR and represent the number of bases upstream from the transcriptional start site at +1 to which each deletion extended. The clone -117 was subsequently used to generate additional mutants by digestion with either Bg1 II + Sst I to create -117 ΔBS or Sst I for -117 ΔS. The DNAs were blunt ended, self ligated, and transfected into the bacterial strain HB101 (BRL). Clone -117 +56 was created by ligating an Sst I to Hind III synthetic oligonucleotide fragment containing the sequences +39 to +56 from the CAP site into the Sst I and Hind III sites of clone -117.

Preparation of Biotinylated Oligonucleotides

Typically 2 nmoles of single-strand oligonucleotide were biotinylated in water, and then annealed to its complementary strand. Photoprobebiotin (1 μg/μl-Vector Labs) was added to the nucleic acid solution in an amount equal to the mass of nucleic acid. The tube was placed in an ice water bath so that the surface of the mixture was 7 cm below the sunlamp, a glass microscope slide was placed over the mouth of the tube, and the mixture was exposed to the sunlamp for 15 minutes. All procedures prior to exposing the reaction to the sunlamp were conducted in a darkened room. The biotinylated strand was annealed to its complementary strand by adding the complementary strand and 1/10th volume of annealing solution. The mixture was placed at 80°C for 2 minutes and then at 37°C for 1 hour. The mixture was adjusted to a pH >8.5 by the addition of 1 M TrisHCl, pH 9.0, and extracted three times with equal volumes of n-butanol. The volume was adjusted to 10 pmole/μl nucleic acid, and then stored at -20°C.

Preparation of Nuclear Extracts

A typical assay contained the proteins extracted from the nuclei of 1 x $10^8$ cells. Lymphoblasts used in this study were maintained at 5 x $10^5$ to 1 x $10^6$ cells/ml in RPMI 1640 + 15% FCS. Six x $10^8$ cells were labeled in 1.0 ml DMEM-met containing 1.5 mCi $^{35}$S-methionine at 37°C for 30 minutes in a 35 mm dish. After labeling, the cells were pelleted by a 30 second spin at 500 x g, washed two times with phosphate-buffered saline (pH 7.3), washed one time in 30 mM Tris pH 8.0, 1 mM KCl and suspended in 1.0 ml of this solution. The cells were disrupted by 10-20 strokes in a 1.0 ml dounce homogenizer (Type B). The crude nuclei were pelleted by a 15 second spin at 1000 x g and suspended in a solution containing 0.1% NP40, 1.5 mM $MgCl_2$ and 420 mM KCl (1.4 ml per 3 x $10^8$ harvested cells). A final concentration of 300 mM KCl was sufficient for extraction of the oligonucleotides. The extraction was carried out at 4°C on a slowly rotating wheel for 1.5 hours. The extract was then centrifuged for 5 minutes at 13,000 x g at 4°C. The supernatant was removed and the pellet rinsed with 1.0 ml of the above solution and recentrifuged. The supernatant was combined with the original volume of extract and the KCl was adjusted to a final concentration of 100 mM. The extract was pre-adsorbed with streptavidin-agarose, by adding 1.2 ml of extract to a 75 µl pellet of agarose beads. Pre-adsorption was for 20 minutes at room temperature. The mixture was centrifuged at 13,000 x g for 5 minutes at room temperature. The supernatant was carefully removed so as to leave 20 µl of supernatant over the pelleted beads.

Binding Assay

The pre-adsorbed nuclear extract was placed in eppendorf tubes at a volume of 1.0 ml. When competitor nucleic acid was used, it was added first and allowed to mix with the extract for 15 minutes at room temperature. The competitor, poly(dI-dC)-(dI-dC) (Pharmacia, Piscataway, NJ), was added at 40 times the concentration of the double stranded oligonucleotide. The amount of oligonucleotide added was 100 pmoles/ml of mix unless otherwise noted. After 20 minutes on a slowly rotating wheel, at room temperature, the mix was centrifuged for 2 minutes at 13,000 x g.

All but 20 µl of the mix was removed and added to 35 µl pellet of streptavidin-agarose. After rotating for 30 minutes at room temperature, the mix was centrifuged for 15 second at 13,000 x g. The supernatant was removed and the beads washed three times with a solution containing 100 mM KCl and 1.5 mM $MgCl_2$. The bound proteins were analyzed by polyacrylamide gel electrophoresis. Immediately prior to loading the gel, the beads were thawed, placed in a boiling water bath for 3 minutes, centrifuged for 2 minutes at 13,000 x g and the supernatant loaded onto the gel.

Nuclear Transcription Analysis of Mitogen Induced Gene Expression

Jurkat T cells were infected and cloned 7 days later. At the time of analysis following 50 days in culture, no RT activity was detectable. Nuclei were isolated after 4 hours of culture in the presence or absence of PHA (1 µl/ml) and PMA (25 ng/ml). In vitro RNA transcription was performed and the $^{32}P$-radiolabeled nuclear RNA was hybridized to cDNAs immobilized on nitrocellulose filters.

Dot-blot Analysis of HIV-1 RNA Levels in Infected Lymphocytes Following Mitogen Activation

$CD4^+$ lymphocytes were purified from normal donors by affinity rosetting, activated with PHA, and infected with HIV-1. Cultures were maintained in RPMI 1640 -10% fetal calf serum supplemented with 10% partially purified human IL-2. Six days after infection, uninfected and HIV-1 infected cultures were washed and incubated with medium or medium supplemented with PHA (1 µg/ml) and PMA (25 ng/ml) for 18 hours. Total cytoplasmic RNA was isolated, serially diluted two-fold, immobilized on nitrocellulose filters, and hybridized to $^{32}P$-radiolabeled tat-III cDNA.

Mitogen Stimulation of the HIV-1 LTR and CAT Assays

Plasmids containing the HIV-1 LTR ligated to the CAT gene (-117 construct) were cotransfected using DEAE-dextran into Jurkat cells (5 µg DNA per $10^7$ cells) with either a tat-I cDNA expression vector or a control expression plasmid containing an inactive frameshift on the

tat-I gene (Δtat-I). Ionomycin (0.5 μg/ml), PHA (1 μg/ml), or PMA (50 ng/ml) was added 24 hours after transfection. Extracts from 2 x $10^6$ cells, normalized for total protein content, collected 20 to 24 hours after induction, were incubated with $^{14}C$-labeled chloramphenicol for 8 hours to determine CAT activity (49). The acetylated and nonacetylated reaction products were separated by a thin layer chromatography. The level of CAT activity was determined by cutting out the regions of acetylated chloramphenicol and counting in a scintillation counter. Results were reported as a ratio of counts induced relative to that obtained from uninduced cells (basal level = 1.0).

Scrape Loading of Recombinant Tat Protein

Scrape loading of recombinant Tat protein was performed as follows. HeLa cells stably transfected with HIV-LTR-CAT (2 x $10^6$ cells/75 $cm^2$ flask) were scraped from flasks using a Teflon scraper and loaded with 10 μg protein extract in buffer containing 140 mM KCl, 10 mM $KH_2PO_4$, and 0.1 M EDTA for 5 minutes at 37°C. Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum was added, cells were lysed by freeze/thawing, and CAT assays were performed on the cell extracts according to Gorman et al. (48).

RNA Gel Mobility Shift Assay

RNA probes were generated by in vitro transcription of truncated templates with T7 or SP6 enzymes in the presence of α $^{32}P$ UTP. RNA probes were purified on denaturing polyacrylamide gels (10%). The specific RNA band was excised, eluted, precipitated in ethanol, and resuspended in $dH_2O$ for gel mobility shift assay.

RNA binding reactions were carried out for 10 minutes at room temperature in 30 μl reactions containing 60 mM NaCl, 12 mM HEPES (pH 7.9), 12 mM DTT, 4 mM Tris (pH 8.0), 4 mM $MgCl_2$, 10% glycerol, 1 μg poly dIdC, 60 units placental RNase inhibitor and 1000-3000 cpm RNA probe. Reaction were mixed with 0.5 x TBE buffer (19:1) and loaded on 8% polyacrylamide gels. Electrophoresis was carried out for 2 hours at 200 volts. Gels were dried and exposed to X-ray film. For Tat 1-72 binding, the reaction (20 μl) contained 50 mM Tris HCl, pH 7.0, 1 mM 2-mercaptoethanol, 500 ng heparin, and 10,000 cpm TAR-RNA. After 30 minutes

at room temperature, glycerol was added and the reactions were electrophoresed for 2 hours at 200 volts on 8% TBE polyacrylamide gels.

Site Directed Mutagenesis

A 0.4-kb SmaI-BamHI fragment containing sequences derived from the tat cDNA clone pCV-1 (20) was excised from pTAT (50) and inserted into the replicative form (RF) of M13mp18. Site-directed mutagenesis was performed with 20-base oligodeoxynucleotides by standard procedures (51). For the gly2,5,9 mutation a 42-base oligodeoxynucleotide was used to generate the triple missense mutant. Mutations were confirmed by DNA sequencing. The XbaI-BamHI fragments from wild-type and mutant RF phage DNA were subcloned into the eukaryotic expression vector $pSV_L$ (Pharmacia, Piscataway, NJ) containing the SV40 late promoter, splice donor-acceptor, and polyadenylation signals. To construct the mutants D2-8Gly9, SH-2, and SH-3, the double-stranded oligonucleotides with XbaI and ApaI ends was subcloned into the unique XbaI and ApaI restriction enzyme sites in $pSV_L$gly2,5,9. The nucleotide sequences of coding strands used to generate these mutants were as follows (coding sequences underlined): CTAGATCGACAGAGGAGAGCAAGAAATGGGGCC (D2-8GLY9), CTAGATCGACAGAGGAGAGCAAGAATGGAGCAACTAGAGCAACTAGGGCC (SH-2), CTAGATCGACAGAGGAGAGCAAGAAATGGAGCAACTAGAGCAACTAGAGCAACTAGGGCC (SG-3). The mutants generated by this procedure are shown in Figure 13. Each of the three acidic residues at positions 2, 5 and 9 were converted to a neutral residue (gly). In other constructs the residues at position 5 or 9 were converted to acidic (asp, glu), neutral (ala) or basic (asp) residues.

## 3. Results

LTR-Directed Expression in Infected and Uninfected Cells

A series of plasmid constructs containing deletions of HIV-1 LTR linked to the chloramphenicol acetyl transferase (CAT) gene were constructed as described in Figure 12. The 5' deletion clones were transfected into H9 cells or H9/HIV cells and CAT assays were performed on the cell lysates within 40-48 hours post transfection. The results of the assays are shown for each

clone in Figure 14. The first column shows values of CAT activity following transfection into H9 cells and the second column shows the results in H9/HIV-1 cells. The much increased level of CAT activity in infected cells is apparent from the reaction conditions. The RSVCAT plasmid (values in parenthesis) served as a positive control in these experiments for both transfection efficiency and CAT expression. The values for infected cells show >1000 fold activity as opposed to uninfected cells for clones containing complete LTRs.

Deletions to position -117 showed no significant differences (<2 fold variation) from CD12CAT in either infected or uninfected cells. However, the activity of clone -103 in uninfected cells was reduced by a factor of 4-9 fold while values for infected cells did not significantly change. Further deletions to -65 and -48 resulted in loss of activity in uninfected cells and either partial or complete loss of activity in infected cells respectively.

Lack of a Negative Regulatory Elements

Negative regulatory elements were not detected in the U3 region in this study in contrast to previous studies (12). The reasons for this are unclear. The 6-fold suppression of activation reported would still allow greater than 1000-fold activity in HIV-1 infected cells relative to uninfected cells reflecting in our opinion a minor role for this element even if it existed.

Localization of Enhancer Elements

The loss of activity in uninfected cells upon deletion of sequences between -103 and -65 suggested that an enhancer element might be located within this region. To test this hypothesis, an oligonucleotide spanning -104 to -80 was synthesized with Xba 1 compatible ends and cloned into the Xba 1 site of -65 and -48. The resulting clones were assayed for functional restoration. The results of the CAT analyses of these clones are shown in Table 13. Clone -65E2 contained the sequences in the 5' to 3' orientation while clone -65E5 contained the reversed orientation. The direct repeats were inserted ahead of -48 in the 5' and 3' orientation in clones -48E9 and -48E14 and in the 3' to 5' orientation in clone -48E8. DNA sequencing confirmed that the clones contained the entire -105 to -80 region except -48E14 which is missing base pair -80.

In all, plasmids -65E2 and -65E5 were positive within or above the range seen for the fully active LTR sequences of CD12 while -65 (data not shown) and the clones -48E8, -48E9 and -48E14 were approximately 10-fold less active. Since the activities seen with these clones did not depend on the orientation of the inserts, the direct repeat region has the property of the enhancer.

The tat Response Region (TAR)

Previous studies have shown that heterologous promoter and enhancer sequences could be placed upstream of the -17 to +80 region of the HIV-1-LTR and be activated 1000-fold (12). We have made further deletions in this region to more precisely define the elements which are responsible for the transactivation phenomenon. For this we used the tat responsive clone, -117, to construct a deletion from the Bgl II site at +20 to the Sst I site at +38. The clone, -117 ΔBS, lacked tat responsiveness but displayed normal levels of expression in H9 cells as shown in Table 13. Thus sequences intermediate of Bgl II and Sst I were indispensable for tat response. Furthermore a deletion of four base pairs of the Sst 1 site GAGCTC at +34 was also made. This clone, -117 ΔS, had no tat response when transfected into H9/HIV-1 cells (Table 13). Identical results were obtained when clones -117 ΔBS or -117 ΔS were transfected into nonlymphoid COS-1 cells.

To further delimit the region necessary for tat response shown here to extend downstream of base pair +38, a 17 base pair oligonucleotide of sequences from +39 to +55 with 5' Sst I and 3' HindIII insertable ends was synthesized and cloned into the Sst I (+38) and HindIII (+79) +55. The resulting clone, -117 +56H was restored for tat response (data not shown). Inspection of the sequences in the region of the Bgl II and Sst I sites for significant features revealed an inverted repeat sequence of 11 and 10 base pairs (bp) at positions +12 and +40. An 8 bp directly repeated sequence (CTCTCTGG) was also present at +5 and +37. Simply preserving the inverted repeat sequences as in -117 ΔBS was not sufficient for maintaining tat responsiveness. The larger stem loop structure may be important because of the spacing between the inverted repeat. On the other

hand, the second CTCTCTGG sequence was disrupted in both -117 ΔS and -117 ΔBS and it is possible that this sequence may be important for tat response.

Characterization of Cellular Proteins that Recognize the HIV Enhancer

Nucleic acid affinity procedures were designed to identify cellular proteins which bind the enhancer element of HIV and may be involved in directing expression of genes linked to this region. For this purpose, we developed a DNA-affinity precipitation assay analogous to immunoprecipitation studies of cellular proteins. The principle is to separate specific DNA binding proteins from complex mixtures of proteins, and then to resolve them by gel electrophoresis. Synthetic oligonucleotides or restriction fragments were covalently modified by the addition of a biotin group and mixed with either whole cell or nuclear protein extracts. The protein-nucleic acid complexes were collected on streptavidin-agarose beads. The proteins were eluted from the beads and resolved by standard gel electrophoresis techniques. A routine assay can be accomplished within 3 hours, excluding gel running time. The assay can therefore be used to directly characterize proteins from individual cell lines that recognize a particular nucleic acid sequence and to compare such proteins to those that bind to other regulatory elements. This assay can also be used to demonstrate cell specific or response specific binding proteins by analyzing different cell lines under various growth conditions. In addition to isolating those proteins that interact with a nucleic acid-protein complex, these assays can be used to identify proteins that bind specifically to the nucleic acid. This simple assay is meant to complement current, widely used procedures such as gel retardation and nuclease protection assays of protein-nucleic acid interactions.

Our earlier studies with deletions of the HIV-LTR have revealed an enhancer element between -105 and -80 base pairs upstream of the transcription start site and both positive and negative control regions at sites upstream of this enhancer element. In continuing these studies, we examined the interactions of cellular proteins with the genetically well characterized HIV enhancer. This enhancer (-104 to -80) contains a 100 bp direct repeat, GGGACTTTCC, that is 100% homologous to enhancers to two DNA viruses, SV40 (52) and human cytomegalovirus (53), and

to a sequence in the kappa-immunoglobulin light chain gene enhancer region (40). For use as probes we synthesized oligonucleotides containing two complete, tandem copies of the region extending from -106 to -79 (Figure 14) (39) which were termed HIVEN3c 1 and 2. Parallel mutant oligonucleotides called HIVEN3m 3 and 4 contain three point mutations within each decamer repeat. The mutant construct was chosen because it had previously been shown to abrogate PHA ± tat III inducible activity of HIV-LTR reporter gene constructs (40). Figure 15, lane 4, shows the pattern of proteins that bind to the wild-type annealed oligonucleotides HIVEN3c1/2 using nuclear extracts prepared from H9 cells. The pattern is complex with proteins of 130 kD, 110 kD, 86 kD, 40 kD, 35 kD, and 28 kD in size being easily detected. These proteins are not detected in control reactions lacking biotinylated oligonucleotides (Figure 15, lane 3). Using mutant probe HIVEN3m3/4 there is a significant decrease in binding of the protein(s) migrating at 86 kD (Figure 15, lane 5); whereas the remaining proteins are still precipitated. Comparison of the protein patterns with these two DNA fragments define four classes of proteins: *(1)* non-specific proteins such as actin (42 kD) that are precipitated by the streptavidin-agarose beads; *(2)* proteins enriched by binding the wild-type fragment and the mutant fragment, such as 130 kD and 110 kD; *(3)* a protein(s) preferentially associated with the wild type fragment, 186 kD; and *(4)* a protein(s) designated by the "*" that bind to the mutant oligonucleotide probe.

The relationship between the LTR binding proteins in stimulated Jurkat cells to those found in H9 cells was compared. Two human B-lymphoblast cell lines expressing exclusively either the kappa- or the lambda-immunoglobulin light chains (54) were also included. The kappa-immunoglobulin producing cell line provides a putative NF-KB positive control for comparison with the PHA-induced Jurkat cells, and the lambda-immunoglobulin producing cell line was chosen to determine if they possess similar and/or unique proteins.

To address this issue we carried out a series of gel-retardation assays using a cloned HIV enhancer 12-base pair probe (-GGGACTTTCCAG-) in addition to the microscale affinity assay. Figure 16 shows that protein extracts from each of the cell lines assayed contained activities that bind to this sequence element. These patterns are more complex than previously reported (55).

The specificities of these interactions were determined by the addition to the retardation reactions of competitor oligonucleotides that were the same biotinylated probes used in the binding assays. Band 1 is found only in H9 cells, not in the B-lymphoblast cell lines nor in the Jurkat cells ± PHA. It is competed specifically by the HIVEN3c1/2. Band 3 (a poorly resolved doublet) is found in H9 cells, in both kappa- and lambda- light chain producing B-lymphoblast lines, and is substantially induced in Jurkat cells upon stimulation with PHA for times previously shown to be sufficient to induce transcription of HIV-LTR linked reporter genes. This behavior correlates with the activity referred to as NF-KB in that lectin stimulated Jurkat cells and kappa-immunoglobulin producing B-lymphoblasts both form the band 3 complex. The lambda-immunoglobulin producing cells also appear to have an identical activity (Figure 16, BN10, band 3). These assays show that an 86 kD protein (Band 3, designated HIVEN86A protein) is present in H9 cells and both B-lymphoblast cell lines. In Jurkat cells, stimulation with PHA results in a significant increase of this protein binding to the HIVEN3c1/2. These results, therefore, have identified a protein of 86 kD which binds to the HIV enhancer that appears following stimulation of Jurkat cells with PHA.

Studies on the Post-Transcriptional Effects of tat

The post-transcriptional effects of tat were examined using a system in which any transcriptional control by tat, is eliminated by looking just at post-transcription events. Plasmids were constructed which contained the T7 RNA polymerase recognition sequences upstream of the TAR region joined to the chloramphenicol acetyl transferase gene in plasmid T7-TAR-CAT as shown in Figure 17. This construct was digested with Bam HI and capped RNA transcripts were synthesized in vitro using T7 RNA polymerase (Figure 18). The resulting transcripts contained bases 1 to 80 of the HIV mRNA linked to the CAT gene as determined by Northern analysis and by primer extension. These RNAs were then microinjected into Xenopus oocytes in the presence and absence of tat expression vectors. Extracts of these oocytes were prepared and analyzed for CAT activity. Co-injection of the HIV TAR-CAT RNA with tat expression vectors resulted in a 30-fold increase in the amount of CAT synthesized relative to that with HIV TAR-CAT RNA

clone. This is direct evidence that the transactivating events mediated by tat III are at least in part post-transcriptional.

Activation of HIV-1 LTR by T-Cell Mitogens and the tat-I Protein of HTLV-I

The enhancement of HIV-1 gene expression by mitogenic stimulation was examined in in vitro RNA transcription assays. Mitogen induced HIV-1 gene transcription was measured in HIV-1 infected Jurkat T cells before and after combined stimulation with PHA and PMA. As shown in Figure 19A, HIV-1 gene transcription, as monitored with tat III cDNA (containing 5'-sequences common to all HIV-1 transcripts) was induced within 4 hours following stimulation. These mitogens also induced transcription of the interleukin 2 receptor (Tac) gene as expected in activated T cells and moderately increased expression of the actin gene. In contrast, the constitutive transcription of the transferrin receptor gene was not altered. With purified CD4+ peripheral blood lymphocytes (PBL) infected in vitro with HIV-1, PHA plus PMA also augmented cytoplasmic HIV-1 RNA as measured by a quantitative dot blotting technique (Figure 19B).

The ability of the mitogens to alter the activity of HIV-1 LTR was measured in an in vitro system in which the LTR was linked to the chloramphenicol acetyl transferase (CAT) gene. For these analyses, a nested series of 5' deletion mutations or full length LTR was linked to the CAT gene and transfected into Jurkat T cells. A single stimulatory agent (PHA, PMA, ionomycin or cotransfection with an expression vector encoding the tat-I protein of HTLV-I) is sufficient to induce interleukin-2 receptor gene expression in these leukemic cells (56,57,58). In contrast, a combination of two of these signals (for example, PHA plus PMA, tat-I plus PHA or tat-I plus PMA) is required to activate interleukin-2 (IL-2) gene expression (58,39).

Each of the T-cell mitogens (ionomycin, PHA, PMA and tat-I) was found to be capable of stimulating the HIV-1 LTR. The results of CAT activation using the -117 deletion mutant (59) of the HIV-1 LTR are shown in Figure 20 as an example of each of these responses. A similar analysis was also carried out with full-length LTR and the complete series of 5' deletion mu ants, to determine the location of the cis-acting LTR sequences responsible for mitogenic activity. The

results of these analyses, expressed as a percent transactivation by full-length LTR is shown in Figure 21.

A deletion of the region located between -671 and -278, resulted in increased basal and mitogen-stimulated HIV-1 LTR CAT activity, suggesting that a negative regulatory element (NRE) is located in this region. Rosen et al. (45) have mapped an NRE between -340 and -185 which affects basal activity. Our finding demonstrate that this NRE also controls the magnitude of the mitogen-induced responses, and suggests that its 3' border does not extend beyond -278.

The maximal changes in PHA and PMA induced LTR activities were observed with the -278 construct. Further deletion to -176 was associated with a decline in this activation while basal activity remained constant. This region (-278 to -176) contains two segments highly homologous to two imperfect repeats within the 5' regulatory regions of both the IL-2 and IL-2 receptor genes (59). Further deletion to -117 and –103 which removes one base from the first direct repeat (enhancer) of the HIV-1 LTR produced a decline in both the basal and PHA and PMA induced responses. Additional deletion to -65, which eliminates both of the direct repeats and one of the three Sp1 binding sites (23), but leaves the "TATA" box intact, was associated with a near complete loss of basal activity and mitogen inducibility.

The stimulation of the HIV-1 LTR by Ionomycin showed very little response to the various deletions, and remained constant at 1.7 to 2.5 times the basal level over the full range of deletions, until -65 where the stimulatory effects disappeared (data not shown). Although these effects were small, they were consistently observed in 21 of 21 determinations using either the full length or deleted versions of the HIV-1 LTR.

Stimulation of HIV-1 LTR by tat-I, the transactivator from HTLV-I infected cells, was also examined in these assays, by cotransfection with a plasmid encoding tat-I. The -671, -278, and -176 deletions exhibited only a modest stimulation by tat-I relative to the basal response, and certainly significantly lower than observed with PHA and PMA. The -117 and -103 deletions, on the other hand, exhibited marked stimulation of the HIV-1 LTR by tat-I.

The tat-III protein of HIV-1 is believed to exert both transcriptional and post-transcriptional effects on HIV-1 gene expression (21,60,61). The potential interplay between the mitogens and tat-III was examined by cotransfecting the Jurkat T cells with both the HIV-1 LTR-CAT (-671 construct) and the tat-III expression plasmid and subsequently inducing with the various mitogens. The amount of HIV-1 LTR stimulation by mitogens alone or in combination with the tat-III was determined, and the results are shown in Table 14. The results are expressed as amount of CAT activity, relative to that produced in medium alone (relative CAT activity equals 1.0). These results show that combinations of the mitogenic agents (ionomycin, PHA or PMA) and tat-III produced synergistic stimulation of HIV-1 LTR. A similar synergistic effect was observed with combinations of tat-I and tat-III (data not shown).

Binding of tat to the 5' Region of Viral mRNA

The interactions between Tat protein and the LTR-TAR regions observed by us and reported by others have suggested a direct binding of the Tat protein to this region. We wanted to determine whether such direct binding can actually be demonstrated. Recombinant Tat proteins were produced in E. coli and baculovirus expression systems. These proteins are biologically active as demonstrated by the scrape-loading procedure into HeLa cells, which contained a stably integrated HIV-1 LTR linked to the bacterial chloramphenicol acetyl transferase gene. For this procedure, the cells are scraped into a low salt buffer, mixed with the Tat protein, and allowed to sit undisturbed for 5 minutes before diluting into complete medium. The chloramphenicol acetyl transferase activity was subsequently tested by standard means.

The plasmid T7-TAR-CAT which contains the T7 RNA polymerase recognition sequences upstream of the TAR region joined to the chloramphenicol acetyl transferase gene (Figure 18)was used to generate capped RNA transcripts in vitro using the T7 RNA polymerase. The 2.8 kb fragment from the Bam HI/Hind III digestion of this plasmid was gel purified and used in in vitro transcription reactions. $^{32}$P-labeled transcripts from the initial 80 nucleotides from the 5'-end of HIV-1 mRNA were generated in this manner.

The RNA gel mobility shift assay was used in conjunction with wild type and mutant transcripts to determine the requirements for the binding activity. A 4bp deletion in the T7-TAR-CAT plasmid was generated by SstI digestion, blunt ending with T4 polymerase and religation of the T7-TAR-CAT plasmid. This procedure removed 4 bp at +35 to +38 nucleotides. The resulting plasmid (ΔS) contained a deletion overlapping the loop and stem, resulting in an enlargement of the loop and the loss of the UCU bulge at +23 to +25 (Figure 22). This mutation had been shown to abolish transactivation of the HIV-1 LTR-CAT (62).

RNA transcripts of the T7-TAR-CAT construct containing the ΔS mutation were prepared and incubated in the presence or absence of the recombinant Tat proteins from the E. coli or baculovirus expression systems (63). The resulting complexes were analyzed by gel shift assays of RNA mobility using polyacrylamide gel electrophoresis. The results of the gel shift assays are shown in Figure 23. Wild-type TAR transcripts formed a stable complex with both sets of recombinant Tat proteins resulting in retarded gel migration. The mutant ΔS transcript, on the other hand, did not form a stable complex (Figure 23A). These results suggest that the (+35 to +38) region of the HIV-LTR is critical to the binding of Tat protein. The deletion of this region may result in the alteration of the loop sequence necessary for the recognition of Tat.

In a similar set of experiments, we examined the ability of the RNA transcripts to compete with the wild type RNA for the binding of Tat. As shown in Figure 23B, the addition of excess amounts of unlabeled transcripts from both wild type and ΔS constructs effectively competed with the wild type RNA for binding to the Tat protein. Similar results were obtained whether the Tat was derived from E. coli or baculovirus. Thus, although ΔS transcripts cannot bind efficiently to Tat protein, they can compete with wild type transcripts for this binding.

The specificity of the gel shift assay for binding to Tat was demonstrated in a similar series of experiments using recombinant gp160 or rev protein produced in a Baculovirus system. These recombinant antigens failed to bind to the TAR transcripts, while the recombinant Tat protein produced in either the Baculovirus or E. coli system demonstrated efficient binding (Figure 23C).

Functional Characterization of tat Mutants - Changes in Acidic Residues

The functional significance of the acidic residues within the amino-terminal region of Tat was examined by means of site directed mutagenesis and by testing the capacity of mutant proteins to transactivate the viral LTR. A series of mutations in the amino terminal of HIV-1 tat was generated and inserted into the expression vector $pSV_L$. The function of these substitutions was determined by cotransfection of these clones with pC15CAT containing the HIV-1 LTR linked to the chloramphenicol acetyltransferase (CAT) gene into COS-1 cells. The relevant nucleotide sequence of the wild type Tat protein and the amino acid substitutions are shown in Figure 13. Single amino acid changes of the acidic residues at positions 2, 5, and 9 to Gly (mutants gly2, gly5, and gly9) were only partially defective in tat activity, as determined by the level of CAT activity expressed from the HIV-1 LTR (Figure 24). A triple mutant converting all three acidic residues to Gly (gly2,5,9) was virtually inactive, suggesting that individual acidic residues might function in an additive manner. The gly2 mutant exhibited a reduced activity (approximately 13-fold) and was the most deleterious of the individual Gly substitutions, suggesting that the glutamate at position 2 is the most essential acidic residue for tat function. Substitution of the aspartate at position 5 with Gly caused a fivefold reduction in tat activity as did the ala5 mutation. Substitution of Lys (lys5) for the aspartate (acidic to basic) resulted in a further (two-fold) decrease in activity when compared with the gly5 and ala5 (acidic to neutral) mutants. Substitution of glutamate for aspartate (acidic to acidic) caused some reduction in tat activity, but was better tolerated when compared with the effects of other mutations at position 5. At position 9, substitution of aspartate for glutamate (asp9) at this position also resulted in full activity. From the Chou and Fasman (64) analysis of the individual Gly substitutions, we could predict that the gly9 mutation would abolish the helix formation, whereas the gly2 and gly5 mutations would have little effect. The ala9 substitution, unlike gly9, should not affect the helix prediction. Therefore, it appears that residue 9 may be important for structure rather than charge.

Replacement of the Amino-Terminal of tat Protein

Acidic amphipathic protein domains are associated with transcriptional activators. Secondary structural analysis of the amino-terminal region of tat suggests that this region could form an amphipathic α helix. To test the role of acidic amphipathic sequences in tat function, we replaced the amino-terminal region with heterologous sequences using synthetic oligonucleotides and a cassette mutagenesis approach. A similar approach was used by Giniger and Ptashne (65) in the analysis of the yeast GAL4 protein. We took advantage of a unique ApaI site introduced by the gly9 mutation to insert synthetic double-stranded oligonucleotides containing both untranslated leader sequences to the translational initiation site and heterologous sequences encoding repeats of the sequence Glu-Gln-Leu, which would form an amphipathic structure if an a helix were to form. The amino acid sequences of these substitution mutants are illustrated in Figure 25A. Mutant SH-2 substitutes amino acids 2 through 8 with two repeats of the sequence Glu-Gln-Leu. SH-3 substitutes this sequence with three repeats. Both SH-2 and SH-3 retain the original gly9 mutation, as does the truncation mutant Δ2-8Gly9.

The induced CAT activity of each of these mutants is shown in Figure 25B. The truncation mutant produced a low, but observable level of transactivation. At 1 μg of DNA, Δ2-8Gly9 induced a two fold increase in the HIV-1 LTR directed gene expression. This is slightly higher than the level obtained with the gly2,5,9 triple missense mutation (1.2-fold). The addition of the heterologous amino acid repeats increased the observed CAT activity approximately fivefold in the case of SH-2 and SH-3, relative to the truncation mutant. At 10 μg of DNA, these substitution mutants induced approximately 30% of the wild-type tat activity. However, this level of activity was achieved by the wild-type tat construct at 0.1 μg of input DNA. The induced CAT activities of the truncation mutant and substitution mutants (SH-2 and SH-3) at 1 μg of DNA were 2% and 11% of the wild-type level, respectively. The transactivation potential of SH-2 and SH-3 at this DNA concentration is one-third that of the gly9 mutant with an otherwise intact amino terminus.

## 4. Discussion

Our studies of the activation of HIV-1 gene expression have focused on the interactions between tat, LTR, and cellular factors in the regulation of genes linked to the HIV-1 LTR. Mutation experiments in the HIV-LTR region have identified an enhancer element of 26 base pairs within the this region. This enhancer is localized at position -105 to -80 from the CAP site. Since the activities seen with the clones in this region did not depend on the orientation of the inserts, the direct repeat region has the property of the enhancer. A perfect homology of the 10 base pair direct repeat was found in the SV40 72 base pair enhancer region in the sense of the late mRNA and in the 18 base pair repeated motif present in the cytomegalovirus major immediate early gene promoter (53). Since the corresponding region of HIV-1 is biologically active, these elements may have a similar biological role in the regulation of cytomegalovirus and SV40 expression as well. Our analyses show that deletion of the first G of the enhancer is not critical since clone -103 is active. Mutational analyses of the homologous sequences in SV40 showed that the last cytosine residue is critical for SV40 enhancer function.

The presence of sequences at -65 were found to be crucial for high level activity along with the enhancer. Sequences within the region are known to bind the nuclear factor Sp-I. The presence of viral encoded potentiators in the H9/HIV cell system (i.e. the tat III protein) may provide a powerful means of measuring very subtle changes in low level transcriptional activity. Alternatively, tat III activation may include an interaction with Sp1 or its target sequence. These possibilities were investigated in cell free transcription systems and DNA binding experiments (see below). The mutation experiments in LTR show the major regulatory elements involved in the region upstream from the HIV-1 promoter consist of an enhancer region from -104 to -80 which acts cooperatively with the Sp1 binding site at -65, site II.

The region downstream of the promoter, the TAR or trans-acting response region is necessary for elevated levels of CAT expression in HIV infected cells relative to that in uninfected cells. The TAR region had been previously mapped to position -17 to +80, but the experiments described here indicate that sequences downstream of +49 are **not** essential for tat response.

It has been demonstrated recently that a major effect of tat activation occurs post transcriptionally (21). We report that any structural feature in the HIV-1 mRNA which would play a role in tat response would have to be located upstream of +55. It is conceivable that the tat protein or cellular factors induced by the virus could bind to such a structure and facilitate post transcriptional enhancement of expression.

Our studies of the mitogen and tat activation of HIV-1 LTR have helped to identify additional functions of specific regions within the viral LTR. These findings raise the possibility of a second NRE located between the -278 and -117 that selectively impairs activation by tat-I. As found with PHA, PMA and ionomycin, deletion to -65 was associated with a complete loss of tat-I activation. Combined stimulation with tat-I and PHA or PMA produced additive effects. In contrast, combinations of PHA and PMA yielded no greater response than that of the most active agent alone.

Our data suggests that, in many respects, the HIV-1 LTR is regulated like a T-cell activation gene. Its response to single mitogenic signals in Jurkat T cells resembles the IL-2 receptor gene (41,42,52). These effects of mitogens on the HIV-1 LTR appear specific since other cellular and viral promoters are unaffected by these stimuli (52).

The tat-III protein of HIV-1 may exert both transcriptional and post-transcriptional effects on HIV-1 gene expression and sequences extending from -29 to +54 within the LTR appear to be involved in this response. To study the potential interplay between the mitogens and tat-III, an expression vector encoding the tat-III gene product was cotransfected with the full length HIV-1 LTR-CAT plasmid into Jurkat T cells. As shown in Table 14, combinations of the mitogenic agents (ionomycin, PHA, or PMA) and tat-III produced synergistic stimulation of the HIV-1 LTR. Similar synergism was observed with combinations of tat-I and tat-III.

The increased HIV-1 LTR activity observed with combinations of the mitogens and tat-III suggest that these agents act through independent mechanisms and underscore the large changes in LTR activity that can occur when both agents are present (for example, > 9000 fold increase in CAT activity in the presence of PMA and tat-III). Thus, even relatively weak mitogenic stimuli,

such as the effect of tat-I on the full length HIV-1 LTR, may lead to marked changes in viral gene expression by triggering production of the tat-III protein.

The capacity of the transactivator gene product of HTLV-I to stimulate the HIV-1 LTR when present in the same cell is similar to the effects of many DNA viruses including herpes simplex, papovavirus, and varicella zoster (67). This finding may be of potential clinical importance in view of the recently recognized high incidence (27%) of dual HIV-1 and HTLV-I infection in some populations of intravenous drug abuse patients (68). Our in vitro results raise the theoretical possibility that vaccination or immunization of HIV-1 infected patients could produce adverse effects by activating the replication of virus.

The studies with Tat binding activity suggest that Tat is an RNA binding protein with some preference for TAR-RNA. A mutation within TAR which abolished transactivation also prevented stable complex formation, suggesting that the binding of Tat to RNA could be a functional step in the transactivation pathway.

It is not yet clear which elements in TAR are recognized by Tat in this assay system. It is possible that the size of the loop and some structural feature of the RNA (e.g., the UCU bulge) are important for binding since the ΔS mutation is predicted to convert the loop and bulge into one loop. The fact that the mutated (ΔS) RNA does not form a complex in the gel shift assay (Figure 23A), but can compete with the binding of wild-type RNA to Tat protein (Figure 23B), suggests that the Tat protein has some affinity for RNA in general, forming transient complexes, and that the Tat-TAR complex is more stable. These results suggest that one step in the Tat activation pathway may involve direct recognition of TAR RNA by Tat protein. Cellular factors may also be required for transactivation and may help stabilize the Tat-TAR interactions. The assay system described here may be a useful tool in dissecting the sequence and protein requirements involved in the Tat response.

As was demonstrated for H9 cells, the use of the binding assay can lead to the identification of specific nucleic acid binding proteins in gel patterns derived from separation of whole cell extracts. The identification and resolution of the specific HIV enhancer binding proteins on

polyacrylamide gels permits the direct determination of the effect any specified manipulation of the cell has on the behavior of each polypeptide. General application of this approach to the study of defined nucleic acid control sequences has the potential of providing substantial biological information both before and after the protein is purified. The binding studies described in this report represents the initial step in the systematic application of the binding assay to define inducible and cell type-specific cellular proteins that interact with nucleic acid motifs determined to be of regulatory significance.

The detection of the inducible HIVEN86A polypeptides in PHA-stimulated Jurkat cells may indicate that they are involved in the enhancement of HIV-LTR mediated transcription previously reported (39,40). The fact that the same protein is detected in a kappa-immunoglobulin producing cell line suggests HIVEN86A is a candidate for NF-KB activity (39,40,41). The demonstration that H9 cells and lambda-immunoglobulin producing cells have the same gel-retardation activity as stimulated Jurkat cells and kappa-immunoglobulin cells extends the cell type and differentiation-stage previously reported for the constitutive expression of NF-KB activity (39,40,41). It may be that HIVEN86A is either completely or partially responsible for NF-KB activity. Purification of these polypeptides is the first step in elucidating the biochemical properties of each HIV enhancer binding polypeptide. The binding assay will provide a very convenient method for monitoring such purification efforts.

The availability of the TAR transcripts which have been generated in the course of this work will permit more defined examination of the post-transcriptional effects of tat. The microinjection experiments using transcripts of TAR linked to the CAT gene have demonstrated that such effects can account for a 30-fold increase in the amount of CAT synthesized. The experiments with the protein binding assays with the in vitro synthesized TAR transcripts will also be very useful in examining the role of cellular proteins in the post-transcriptional activities mediated by tat.

Our studies of the acidic amino-terminal region of HIV-1 Tat protein have demonstrated that this region represents an essential activating domain. The region consisting of 13 amino-terminal residues has features such as acidity and potential amphipatic helicity common to eukaryotic gene

activator proteins. Our mutation studies have demonstrated that this region is critical for efficient Tat function.

Although the substitution of the acidic amino acid residues by heterologous sequences expected to form an acidic, amphipatic helix can partially restore Tat function, the level of Tat activity is low. This suggests that some component of the amino acid sequence or some structural specificity may be required, in addition to the acidic and amphipathic character of the region, for full Tat activity. We cannot exclude the possibility that the secondary or tertiary structure of the amino-terminal domain of these cassette insertion mutants differs from that of the wild-type Tat protein. For example, the acidic residues may not be presented in the spatial orientation most favorable to trans-activation. It is also possible that the Gly residue retained at position 9 has deleterious effects.

The results presented here suggest that the amino-terminal domain of HIV-1 Tat is important for transactivation. However, since a detectable level of transactivation is observed in the amino-terminal truncation mutant, we suspect that another region of Tat may also have some activation function.

C. MOLECULAR STUDIES OF THE HIV-1 AND HIV-2 GENOMES

## 1. Rationale

Human immunodeficiency virus types 1 and 2 (HIV-1, HIV-2) are related retroviruses and are found to be associated with the acquired immunodeficiency syndrome (AIDS) in humans (1,68-72). Similarly, a simian immunodeficiency virus, $SIV_{MAC}$ has been isolated from captive macaques with AIDS and was found to be more related to HIV-2 than to HIV-1 (73,74). Other viruses of the same family have been isolated from captive and wild Old World Monkeys (75-79). Genetic comparisons of HIV-1, HIV-2, and SIV revealed that all three retroviruses share common biological and genetic features (29,30,80). The genetic structures of these retroviruses are more complex than the structure of other animal retroviruses. In addition to the structural genes encoding for core and envelope proteins, several accessory genes have been discovered. The tat gene encodes for a 14K Da protein that acts in trans to increase the expression of viral genes (20). The rev gene encodes for a 19 Da protein which differentially regulates the expression of virion protein (22,50,81). The nef gene (27K Da) reduces viral expression while the vif gene is essential for infectivity of cell-free virus (9,34). The vpr gene function is unknown (82). Another gene, vpx, is present in HIV-2 and SIV but not in HIV-1 (83,84). The vpx gene is associated with mature virions, but does not have a known function. A number of these accessory genes are not required for virus replication as revealed in studies involving infection of T-cells in vitro. An in vivo study of the functional roles of accessory genes is required in order to understand the pathogenesis of the AIDS virus.

In 1986 the genetic diversity of the HIV-2 and SIV genomes was unknown. Since the pathogenicity and evolution of these viruses were being debated, a critical issue was to determine the degree and manner of divergence among various HIV-2 and SIV isolates. In order to determine the relationship between HIV-1, HIV-2 and SIV retroviruses, a variety of isolates were molecularly cloned and sequenced during the course of this project. This work has aided researchers in the development of an evolutionary tree for HIV and SIV retroviruses. Studies on

the function of accessory genes were undertaken in order to try to understand the pathogenesis of the virus. With increased knowledge of the molecular structure and biological functions of the HIVs, a need arose to develop an animal model system for studying the divergence and pathogenesis of the virus in vivo. A molecularly cloned HIV-2 virus stock was generated, characterized, and used to infect Rhesus macaques in an attempt to generate a suitable animal model for studying the infectivity and mutational rate of human immunodeficiency viruses. Such an animal model study may also shed some light as to the functional roles of the accessory genes in vivo. A well characterized animal model system can be exploited to study the prevention of infection and the modulation of disease progression during drug therapy or the application of a vaccine. Such studies are needed in order to combat the spread of AIDS.

## 2. Experimental Methods

### Virus Propagation

$SIV_{MAC}$, HIV-$2_{SBL6669}$, HIV-$2_{ISY}$, and HIV-$2_{NIH-Z}$ were grown in the human T cell line Hut-78. Viral production was determined by the level of reverse transcriptase (RT) activity in infected cells.

### Infection of Target Cell Lines

Concentrated virus was obtained from the transfected cell line Hut-78 constitutively producing the HIV-$2_{ISY}$ virus or other HIV-2 and SIV viruses. The equivalent of 1000 $TCID_{50}$ (tissue culture 50% infective dose) infecting virus was used to infect the cell lines H9, Molt-3, U937, Hut-78, CEM, MT-2, and the T-cell clone 55. Cells ($5 \times 10^6$) from each culture were treated with Polybrene (Sigma) at 5 μg/ml for 1 hour. The cells were washed with 1X PBS and incubated for 1 hour with the virus. At the end of the incubation period, the cells were washed with 1X PBS and resuspended in RPMI medium-10% Fetal Calf Serum (FCS). Immunofluorescence and RT activity were measured every 3 days. Cell viability was calculated by

dividing the number of cells not incorporating trypan blue color by the total number of cells. The number of syncytia was counted under a light microscope.

Molecular Cloning of Proviral DNA

High molecular weight DNA was purified from Hut-78 cells infected with HIV-$2_{NIH-Z}$, HIV-$2_{SBL6669}$, and HIV-$2_{ISY}$. Similarly, genomic DNA was purified from CEM•SS cells infected with HIV-$2_{ISY}$ virus obtained 1 and 5 months post-infection in Rhesus macaques. DNAs were partially cleaved with BamHI or Sau3A. Each DNA was size selected by fractionation on a linear 10-40% sucrose gradient and concentrated by ethanol precipitation. The DNA was ligated into the arms of EMBL-3 vector cleaved with BamHI (85). The ligated phage DNA was packaged in vitro for production of a lambda library. Recombinant phages were plated and screened using $^{32}$P-labeled SIV or HIV-2 DNA probes. The HIV-$2_{NIH-Z}$ and HIV-$2_{ISY}$ clones were isolated from their respective library using the SIV gag (pB16) and env (pSS35) probes (86).

The proviral clones for SIV were isolated from a genomic library constructed from the DNA obtained from SIV infected cell line K6W. Nine clones containing overlapping portions of the $SIV_{MAC}$ genome were purified and characterized by restriction endonuclease analysis. Five genomic clones were selected for further analyses.

Desired regions of HIV-2 and SIV were subcloned into Stratagene's Bluescribe or Bluescript vectors (KS$^-$), according to standard procedures (85). Phage DNAs were digested with BamHI, KpnI, or desired restriction endonuclease and fractionated by electrophoresis through 0.7-1.0% agarose gels. Desired fragments were excised and purified. Bluescribe or bluescript KS$^-$ plasmids were digested with desired restriction enzyme and treated with bacterial alkaline phosphatase (Bapped) for removal of 5' phosphates in order to prevent self ligation (85). Equal molar quantities of insert and vector DNAs were ligated at 14°C overnight. The ligated DNA was used to transform AG-1 or DH5α cells by a modification of the Hanahan procedure (87). Transformed cells were selected on ampicillin plates.

Southern Blot Analysis of HIV-2 Infected Cells

The total cellular DNAs from cell lines infected with HIV-$2_{SBL6669}$, HIV-$2_{NIH-Z}$, and SIV were digested with BamHI, XbaI, and/or EcoRI and electrophoresed on a 0.8% agarose gel. As a further control, the DNA of the $SIV_{MAC}$ infected cells was cleaved with the same restriction enzymes. The gel was denatured, neutralized, blotted onto nitrocellulose filters according to standard procedures (85,88). The filters were hybridized to $^{32}$P-labeled SIV probes (pB16 and/or pSS35) or $^{32}$P-labeled BamHI fragments of HIV-$2_{ISY}$ for detection of SIV and HIV-2 sequences.

DNA Sequencing

DNA restriction fragments of the provirus were removed from the genomic phage clones and were subcloned into the appropriate enzyme sites of the plasmid vector Bluescribe or Bluescript. Other subclones were generated by inserting DNA restriction enzyme fragments of HIV-$2_{NIH-Z}$ and $SIV_{MAC}$ into M13 bacteriophages, mp8 and mp9. The DNA sequence was obtained by the dideoxy chain termination procedure using synthetic primers, the Klenow fragment of E. coli polymerase and the T7 DNA polymerase on single and double stranded DNAs (89). Sanger sequencing reactions were performed on Terasaki microsample plates. $^{32}$P-labeled dATP was used in the Sanger reactions. Approximately, 3 KB of the HIV-$2_{NIH-Z}$ proviral DNA and 90% of HIV-$2_{ISY}$ were sequenced by the chemical degradation method of Maxam and Gilbert (90). DNAs from all sequencing reactions were size fractionated on 8% polyacrylamide gels at 1800 volts/30 watts for 2 1/2 and 6 1/2 hours. For sequence readings over 350 bases, 90 cm 6% polyacrylamide gels were run at 2800 volts for 28-45 hours. The nucleotide sequences of each viral clone were analyzed by use of computer program Microgene and IBI's Pustell Sequence Analysis Programs.

DNA Synthesis

All primers were synthesized on an Applied Biosystems Model 381A machine using B-cyanoethyl phosphoramidite chemistry. Primers were purified by Nensorb chromatography (NEN).

Transfection in Neoplastic T Cells

Ten million Hut-78 cells were used for each transfection. Forty million cells were resuspended in 40 ml of RPMI 1640 medium with 10% fetal calf serum (FCS)(GIBCO), and incubated at 37°C for 5 hours. After incubation, the cells were washed with RPMI 1640 medium without FCS and aliquoted (10 million cells) into four tubes. The cells were resuspended in 4 ml of RPMI 1640 medium without FCS containing 50 mM Tris-HCl (pH 7.4) plus 10 μg of DNA. Subsequently, 1 ml of 5X DEAE dextran solution (25 mg/ml) in RPMI 1640 medium without FCS in 1 M Tris•HCl (pH 7.4) was added to each tube. The samples were incubated at 37°C for one hour with gentle shaking. After incubation, the cells were pelleted at 1500 rpm and washed twice at room temperature with complete medium (RPMI 1640 medium with 10% FCS). The following day, 10 ml of fresh medium was added. Viral production was monitored by testing for magnesium-dependent reverse transcriptase (RT) one week post-trasfection. For the RT assay, the proteins contained in the supernatant were precipitated with 30% PEG/0.4 M NaCl and the pellets were resuspended in VSB (91). Reverse transcriptase activity was determined by precipitable counts of incorporated $^{3}$H-thymidine.

Radioimmunoprecipitation

Hut-78 cells infected with HIV-2 or $SIV_{MAC}$ were incubated in methionine and cysteine free medium supplemented with [$^{35}$S]-methionine and [$^{35}$S] cysteine (100 uCi/ml) for four hours and pelleted. In order to obtain the virus and clarify the medium, the supernatant was centrifuged at 26,000 rpm for 1 hour which resulted in the pelleting of the virus. The labeled viral lysate was precleared overnight by incubation with normal human sera and Sepharose bound protein A. Labeled proteins from the medium were reacted with HIV-2 infected or SIV infected sera, diluted 1:100. The immunocomplexes were detected using sepharose staphylococcal protein-A and fractionated by electrophoresis on a 10% SDS-polyacrylamide gel. The gel was treated with enhancer for 30 minutes, dried, and autoradiographed (92).

Immunofluorescence

The expression of surface membrane CD4 and CD8 antigens were determined by direct immunofluorescence assay on live cells using phycoerythain-conjugated Leu 3A (anti-CD4) and fluorescein-isothiocyanate-conjugated Leu 2a (anti-CD8) monoclonal antibodies.

Immunofluorescent staining of infected cells was performed with serum from individuals infected with HIV-2. The cells were pelleted, fixed with 50% methanol/50% acetone for 10 minutes, and incubated with 15 µl of human serum diluted 1:40 with phosphate-buffered saline (PBS) for 30 minutes at room temperature. The slides were washed with PBS and incubated with fluorescein conjugated anti-human antibodies in the dark for 30 minutes. After washing in 1X PBS three times, positive cells were scored under a Leitz fluorescent microscope (92). Electron microscopy on the infected cells was performed as described by Biberfeld et al. (93).

Inoculation of Monkeys with HIV-2

Four 1-year old Rhesus macaques (#172, 173, 176, 177) and two 3-year old African green monkeys (C464, C497) were inoculated intravenously with approximately $10^4$ $TCID_{50}$ of HIV-$2_{ISY}$ (#176, 177, C464) and HIV-$2_{NIH-Z}$ (#172, 173, C497). Two additional Rhesus macaque and one African green monkey (#178, 179, C498) were inoculated with saline as negative controls. All animals were housed in individual cages in the same room. All animal experiments were performed by Dr. Philip Markham at Advanced Bioscience Laboratory, Inc., Kensington, Maryland and Dr. Genoveffa Franchini of the National Cancer Institute.

Western Blot Analysis

Cells from infected cultures were pelleted. Virus was pelleted from the supernatant by centrifugation at 20,000 rpm for 1 hour. The viral pellet was resuspended in 1X RIPA buffer for disruption (5mM phenyl methyl sulfonyl fluoride, 5 mM NaCl, 25 mM Tris HCl, pH 7.5, 0.5% SDS, 5% Triton X-100, 5% deoxycholic acid). The disrupted proteins were loaded onto a 10%

SDS-polyacrylamide gel for size fractionation by electrophoresis and transferred to nitrocellulose (94). The filters were reacted with 5% dry milk in 1X PBS for 1 hour for blocking. Strips were individually reacted with sera obtained from animals at 1:100 dilution. Iodinated *Staphylococcus aureus* protein A was used to detect immunocomplexes in some experiments.

ELISA

Total virions from HIV-$2_{NIH-Z}$ were purified on a sucrose gradient (95) according to standard procedures. Virions were lysed and bound to 96 well microtiter plates. The plates were reacted with various dilutions of monkey sera and the immunocomplexes were detected using goat anti-human antibodies conjugated to horseradish peroxidase (91).

Peptides PT-1 and PT-2 and Rabbit Antisera

The peptides used in this study were obtained from Cambridge Biochemical Corporation and were synthesized using solid-phase peptide synthesis technology. Rabbit antisera were obtained from rabbits immunized four times with peptide PT-1 coupled to keyhole limpet hemocyanin (KLH).

Immunoblot Assay

For detection of antipeptide immune reactivity, human and monkey sera were diluted 1/100 and used against 1 µg of each peptide spotted onto nitrocellulose filters. Bound antibodies were detected using $^{125}$I-labeled *Staphylococcus aureus* protein A at 5 x $10^5$ cpm/ml 5% dry milk. For Western blot assay, 1 x $10^7$ cells infected with HIV-1, HIV-2 or $SIV_{MAC}$ or virions were lysed in 500 µl of radioimmunoprecipitation assay (RIPA) buffer (95) and 50 µl were run on an SDS-PAGE gel (12.5%)(96). After electrophoretic transfer (60 V overnight) to nitrocellulose filters, the samples were reacted with the rabbit anti-PT-1 sera (1/50 diluted) and stained with $^{125}$I-*Staphylococcus aureus* protein A. The molecular weights of reactive bands were calculated relative to the migration of the Amersham "rainbow" markers.

## 3. Results

a) GENETIC ANALYSIS OF SIV AND HIV-2 GENOMES

The immortalized human T-cell line, Hut-78 was infected with HIV-2 viruses, SBL6669 (ISY) and NIH-Z and $SIV_{MAC}$ virus (K6W78). Genomic DNAs were purified from virally infected cell lines and used in the construction of recombinant lambda phage libraries. Positive clones were selected using SIV or HIV-2 probes. The HIV-$2_{NIH-Z}$ virus isolate was obtained by Dr. D. Zagury from a patient with immunodeficiency from Guinea Bissau and was delivered to J. Zagury at the NCI. The SBL6669 (ISY) isolate was obtained from a Gambian individual with AIDS (97). The SIV strain K6W78 was obtained from Dr. Phyllis Kanki and was received by Dr. Genoveffa Franchini of the NCI. The genomes of HIV-$2_{NIH-Z}$, HIV-$2_{ISY}$ and $SIV_{MAC}$ (K6W78) are 9431, 9636, and 9870 base pairs long, respectively. The endonuclease restriction maps of the $SIV_{MAC}$, HIV-$2_{ISY}$, and HIV-$2_{NIH-Z}$ genomes were derived from the lambda phage clones and are depicted in Figures 26 & 27. The overall homology at the nucleotide sequence level between $SIV_{MAC}$ and HIV-1 is 55%. The overall homology of the HIV-2s to HIV-1 is 65%, whereas they share 86-89% homology among themselves. The overall genomic organization of all three retroviruses is identical to other HIV-1, HIV-2, and SIV isolates (Figure 28). This organization consists of 5' long terminal repeat (LTR)-gag-pol-central region-env-nef-3' LTR. A single structural difference lies in the presence of an extra open reading frame (vpx) in the middle of the genome of HIV-2 and SIV (83,84). This reading frame is absent in the HIV-1 genome. Furthermore, all three genomes contain the vpr gene which is present in HIV-1 but not in $SIV_{AGM}$ (98). The HIV-1 genome has an open reading frame, vpu which is not present in the HIV-2 and SIV genomes.

The Long Terminal Repeat (LTR)

All three genomes are flanked by LTR sequences which are known to regulate the viral gene expression. Sequence comparison of the LTR's for each of the three viral sequences with

HIV-$2_{ROD}$ showed the presence of all of the regulatory elements, such as the TATA box, the polyadenylation signal AATAAA, core enhancer sequences, Sp1-binding sites, and the tat-responsive region. Such sequences are highly conserved. The sizes of the LTRs for each of the three retroviruses vary from 632 to 800 bases. The size of the individual components was derived by a comparison of the LTRs of SIV, HIV-$_{NIH-Z}$, and HIV-$2_{ISY}$ with the LTR of HIV-$2_{ROD}$ (Table 15). A sequence alignment of the U3 region of the HIV-$2_{NIH-Z}$ showed a deletion of 228 nucleotides in the HIV-$2_{NIH-Z}$ U3 region. This deletion occurred 60 nucleotides 3' of the polypurine tract (Figure 29). Since this deletion is present in both the 5' and 3' LTRs, it is probably present in the provirus and is not a cloning artifact.

The gag Gene

Retroviral core proteins are derived from the proteolytic cleavage of a polypeptide precursor encoded by the first open reading frame at the 5' end of the genome. In infected cells the size of the gag precursor protein appears to be 55 Kd as judged by immunoprecipitation with human sera from infected individuals (74). The gag open reading frames for HIV-$2_{NIH-Z}$, $SIV_{MAC}$, and HIV-$2_{ISY}$ encode for proteins having 519, 506, and 521 amino acids, respectively. The length of the gag precursor polypeptide reported for different HIV-1 strains varies between 500 and 512 amino acids. The amino acid homologies of HIV-$2_{NIH-Z}$ gag precursor polypeptides when compared with HIV-$2_{ROD}$, $SIV_{MAC}$, and HIV-1 are 92%, 82%, and 52%, respectively (Table 16). Such homology indicates that the HIV-2s are more closely related to each other than to $SIV_{MAC}$. Furthermore, $SIV_{MAC}$ and HIV-2 are more closely related to each other than to HIV-1. The cleavage site for the major core protein (p24-p28) in the HIV-$2_{NIH-Z}$ and HIV-$2_{ISY}$ gag precursors were assigned by alignment of the amino acid sequences of the sequences of the gag precursor polypeptides of HIV-$2_{ROD}$, HIV-1, and $SIV_{MAC}$ (Figure 30).

These alignments were performed using the algorithm of Dayhoff and colleagues. These sequences were aligned with those of the major gag proteins of Visna and EIAV in order to establish their respective phylogenetic relationship (Figure 31). The results expressed as

percentage of amino acid sequence are summarized in Table 17. The analysis of the p24-26 proteins indicates that HIV-$2_{NIH-Z}$ and HIV-$2_{ROD}$ are highly related (96%) and are equally distant from $SIV_{MAC}$ (88%). Both HIV-2 and $SIV_{MAC}$ appear to be equally distant from HIV-1 (66-68%). Equal homology (28-29%) is found between EIAV and HIV-1, both HIV-2 isolates, and $SIV_{MAC}$. Similarly, Visna virus shares only 24-26% homology with HIV-1, HIV-2 and $SIV_{MAC}$. Thus, the comparison suggests an ancestral relationship between the ungulate retroviruses and the primate immunodeficiency viruses, but also indicates that the divergence of the primate immunodeficiency viruses occurred much later while HIV-2 and $SIV_{MAC}$ diverged later still.

At the carboxy terminus of the HIV-$1_{IIIB}$ gag precursor two repeated sequences have been described. The first, an imperfect repeat encoding 12 amino acids, is also present in the $SIV_{MAC}$ genome (Figure 32), as well as in the HIV-$2_{ROD}$ genome at positions 1605-1641 and 1667-1703, suggesting that this sequence duplication must have occurred long ago in a common ancestor of these three viruses. The second, present in HIV-$1_{IIIB}$, is a perfect repeat that also encodes 12 amino acids (99), and is absent in the $SIV_{MAC}$ genome. The fact that this repeat in HIV-$1_{IIIB}$ is perfect and that some other HIV-1 isolates as well as $SIV_{MAC}$ lack it, strongly suggests that the duplication in the HIV-$1_{IIIB}$ genome must have occurred relatively recently. Interestingly, an imperfect direct repeat of 18 amino acids can be detected in the HIV-$2_{ROD}$ genome, indicating that the border between the gag and the pol open reading frame may be particularly prone to duplications.

The pol Gene

The second large open reading frame found in these retroviral strains encode for RNA polymerase (pol). The $SIV_{MAC}$ genome has an open reading frame which encodes for 1053 amino acids whereas that of HIV-$2_{NIH-Z}$ and HIV-$2_{ISY}$ encodes for 1190 amino acids (Figures 29, 32 and 33). The pol gene overlaps with the gag precursor open reading frame as in HIV-1. The

overall amino acid sequence relationships among the pol genes of both HIV-2 isolates, HIV-1 and SIV are like those of the gag genes and lead to the same conclusions.

The Envelope (env) Gene

The third major open reading frame in the HIV and SIV retroviruses encodes for the envelope protein. This is the largest open reading frame in the 3' region of the genome. The env genes of HIV-$2_{NIH-Z}$, HIV-$2_{ISY}$, and $SIV_{MAC}$ encodes for 856, 837, and 881 amino acids, respectively. A comparative analysis of the envelope protein of HIV-$2_{NIH-Z}$ with HIV-$2_{ROD}$ and $SIV_{MAC}$ showed an overall homology of 80% and 70% respectively. The envelope protein of HIV-$2_{ISY}$ also exhibited an 80% homology with HIV-$2_{ROD}$ (Table 18). The degree of conservation is comparable in the extracellular and transmembrane envelope proteins. The envelope proteins of HIV-$2_{NIH-Z}$ and HIV-1 are much less related (35%), as shown in Table 16. A homology comparison between HIV-1 and HIV-2 was undertaken in order to identify conserved regions which may be crucial for the function of the envelope protein. The position of the cysteines is highly conserved among all these retroviruses. In the extracellular envelope protein 22 cysteine residues are conserved among both HIV-2 isolates and $SIV_{MAC}$ and 19 and 22 are also conserved in the same position in all strains reported of HIV-1 (Figure 34).

Similarly in the transmembrane portion of the envelope protein three cysteines are conserved among both strains of HIV-2 and $SIV_{MAC}$ and two are also conserved in the HTLV-IIIB strain of HIV-1. Clearly, disulfide bonds must play a crucial role in maintaining the secondary structure of the envelope proteins.

Further analyses of the amino acid homology among the envelope proteins, identified regions in which either complete amino acid identity or only conservative changes could be detected in all these viruses. Of these envelope conserved regions (ECR) indicated in Figure 34, the ECR-6 which is located in the extracellular glycoprotein has been identified as a putative binding site to the CD4 molecule which is an essential part of the cellular receptor for HIV-1, $SIV_{MAC}$ (100-102) and most likely HIV-2. A peptide (PT-1) capable of inducing cell mediated immunity in mice has been

synthesized using the sequence of the ECR-6 region from HIV-1 (103). Regions ECR-7 and ECR-8 which are located at the amino terminus of the transmembrane envelope protein may represent regions involved in the repetitive folding of the protein within the cellular membrane, as proposed by computer assisted analyses of the envelope proteins of different strains of HIV-1 (104). Based on the homology of the ECR-7 region with the fusion peptide of human paramyxoviruses, measles, and respiratory syncytial viruses (105), it has been hypothesized that the first 11 amino acids conserved in ECR-7 may be the fusion peptide of HIV-2, HIV-1 and $SIV_{MAC}$. Finally, the 3' most conserved region in the transmembrane envelope protein (ECR-12) has been implicated to be involved in the cytopathic effect in vitro even though others (Sodroski et al., 1986) have reported contrary results (106). Despite these differences, which may be related to the use of different target cells, the conservation of these 17 amino acids in HIV-1, HIV-2 and $SIV_{MAC}$ may be biologically significant.

Since $SIV_{MAC}$, like HIV-1 and $HIV\text{-}2_{ROD}$, binds to the CD4 protein, it is possible that the CD4 protein may bind to highly conserved regions. The region containing the last cysteine in HIV-1 gp120 is critical for binding to the CD4 molecule. This region spans from amino acid 442 to 463 in the $SIV_{MAC}$ envelope and has an amino acid sequence almost identical to that in HIV-1 and HIV-2. Another important antigenic site has been mapped in this highly conserved region. Cease et al. (103) have identified two peptides, T1 and T2, which elicit T-cell immunity. The 16 amino acids T1 peptide maps within the putative envelope region binding site of the CD4 molecule.

In $HIV\text{-}2_{NIH\text{-}Z}$ infected cells, a protein of 33 Kd (compared to 41 Kd in HIV-1 infected cells) believed to be the transmembrane envelope protein, has been identified. Similarly, a truncated form of the transmembrane protein has been identified in $SIV_{MAC}$ infected cells. The env gene of $SIV_{MAC}$ contained a termination codon that would eliminate the last 146 amino acids at the carboxy terminus of the transmembrane portion of the envelope (25,107). $HIV\text{-}2_{NIH\text{-}Z}$ and $HIV\text{-}2_{ISY}$ do not have a termination codon at the same position. Since the $HIV\text{-}2_{NIH\text{-}Z}$ infected cells appear to express a truncated gp33, the provirus obtained in our laboratory is not representative of the majority of the provirus present. This stop codon is present in the same position in some

clones of HIV-$2_{ROD}$ (30). A comparison of the amino acid sequence of the transmembrane envelope protein of these viruses, shows a decrease in homology after the stop codon (Figure 35). The presence of a premature stop codon is due to the propagation of the virus in tissue culture (31) and is of no biological significance. A clone which does not contain this premature stop codon will be used for infectivity studies.

The same regions that have been found to vary most in HIV-1 (108) are generally the most variable in both HIV-2 isolates and $SIV_{MAC}$ (Figure 31). The first region of variability among the envelope proteins of HIV-$2_{NIH-Z}$, HIV-$2_{ROD}$ and $SIV_{MAC}$ spans from amino acid 112 to 190 in HIV-$2_{NIH-Z}$ and corresponds to the hypervariable region from aa 130 to aa 210 in HIV-1 (108,109). The degree of variability in this region of the West African retroviruses, $SIV_{MAC}$, and HIV-1 isolates were analyzed. The percentage of amino acid identity ranged from 30-60%. This equivalence suggests that both groups of viruses may have spread to their present ranges from a limited focus of infection at approximately the same time.

Identification of Putative Functional Domains of Other Viral Proteins

Several other genes have been identified in the HIV-1 genome by immunological (81,82,110,111) or functional studies (20,22,81,106). The corresponding genes can be identified in the HIV-2 and SIV genome and the comparative analyses of their amino acid sequence indicate strongly conserved domains within some of them (Figure 28).

The HIV-2 rev gene, which was discovered in HIV-1 by mutagenesis of a biologically active HIV-1 clone (22,81), seems to be crucial for the expression of the HIV-1 envelope protein. Protein sequence alignments of the HIV-2 and HIV-1 rev gene products show an arginine rich region in the second coding exon that is conserved among HIV-1, HIV-2 and $SIV_{MAC}$ (Figure 36). Similarly, arginine and cysteine rich regions (Figure 36) can be identified in the first coding exon of the tat proteins, which are responsible for the transactivation of virus expression in these viral isolates (70,112). No recognizable conserved regions were detected within the vif gene although the vif proteins of these viruses share a similar hydropathy profile (not shown). A highly

conserved region could, however, be identified in the nef gene although a correct protein alignment of the nef protein product can not be obtained because of the presence of the 228 nucleotide deletion in the U3 region of HIV-$2_{NIH-Z}$ (113).

Studies of the vpx Region

In continuing our analysis of the comparison of the HIV-1 and HIV-2 genomes, we have focused attention on a portion of the HIV-2 genome containing an open reading frame (designated vpx) which does not have a counterpart in HIV-1. To establish whether vpx is a gene, we studied its expression in HIV-2 infected individuals and in infected cells in vitro. An HIV-2 proviral DNA fragment containing the vpx was expressed in E. coli and the recombinant protein was used in an immunoblot assay. The vpx protein was recognized specifically by the sera of HIV-2 infected people but not by the sera of SIV infected monkeys or HIV-1 infected humans. A rabbit antiserum raised against the recombinant vpx protein recognized a 16 Kd protein in HIV-2 infected cells (Figure 37).

Molecular analysis of the native vpx protein revealed that the protein was not glycosylated or phosphorylated. The protein was localized predominantly in the cytoplasm of HIV-2 infected cells. This HIV-2 p16 appears to be associated with the mature virion, but we do not know at present whether the protein is packaged inside the viral particles or if it is associated with the envelope of the virus during the budding process.

In summary, we identified a novel gene product of HIV-2 (p16) and generated reagents that may be used as diagnostic reagents as well as help elucidate the function of the p16 in HIV-2 infection and pathogenisis. The fact that the sera of HIV-1 infected people do not recognize the recombinant vpx protein in a Western blot assay could be exploited to generate a sensitive assay to discriminate between people infected with HIV-1 or HIV-2.

In Vivo Expression of the SIVMAC Carboxy Terminus of env

Since the carboxy terminus of the envelope transmembrane protein has been implicated in the cytopathic effect of HIV-1 in vitro, we decided to investigate whether putative expression of the open reading frame located after the termination codon correlates with the pathogenicity of SIV in vivo. Two synthetic peptides were generated from the inferred amino acid sequence of SIV and tested for reactivity by Western blot against the sera of naturally and experimentally infected monkeys as well as against sera of HIV-2 infected individuals. Results indicate that the protein synthesized from this open reading frame is expressed in vivo, since an immune response can be detected against the synthetic peptides in 2 out of 3 experimentally infected animals. However, no correlation can be found between its expression and disease progression at this time. Furthermore, a rabbit immune serum raised against the synthetic peptide failed to identify any specific protein in SIV infected cells.

Based upon the hydropathy profile of the inferred amino acid sequence downstream from the termination codon (position 746-890), two hydrophilic regions were chosen for generating synthetic peptides (Figure 38). The first peptide, THTQQDPALPTREGKEGDG, is located at the amino terminus and was designated PT-1 while the second, LRRIREVLRTELTY, was designated PT-2 (Figure 38). The peptides were synthesized using solid phase peptide synthesis technology by Cambridge Biochemical Corporation. The synthetic peptides (1 ug each) were bound to nitrocellulose and reacted in a Western blot assay with sera obtained from various animals. An unrelated peptide of equivalent molecular weight was used as a negative control (WSKMDQLAKELTAE). Sera were reacted with nitrocellulose strips in a 1:100 dilution and the immune complexes were detected using iodinated *Staphylococcus Aureus* protein-A of $5 \times 10^5$ cpm/ml in blotto reagent (94). Sera obtained from animals (Table 19) which scored positive when tested by Western blot using SIV viral proteins were tested along with animals that scored negative in the Western blot assay.

Both synthetic peptides were recognized by serial sera obtained from a macaque (6325) 2 months after experimental infection with SIV. As shown in the lower part of Figure 39, no

reactivity was detected in the sera obtained before the inoculation of the animal and 1 month after inoculation. Reactivity against PT-1 and PT-2 was detected two months after inoculation and lasted to the death of the animal from immunodeficiency 17 months later. Animal 6324 recognized only one of the peptides (PT-1) (lower part of Figure 39). The immune response against PT-1 also lasted till death from immunodeficiency 18 months later. In both macaques the reactivity against the synthetic peptides was coincident with the onset of antibodies against the major gag protein (p24-26) and the putative truncated transmembrane portion of the envelope protein (gp32-34) (upper part of Figure 39). Animal 6325 also developed detectable antibody response against the extracellular portion of the envelope protein (gp120). In contrast, animal 6324 exhibited a lower titered immune response against the gp120. The third macaque (6323) sero-converted one month after SIV inoculation and developed a strong immune response to both the envelope glycoproteins and the gag proteins, but its serial serum samples did not recognize the peptides PT-1 and PT-2. However, the 6323 animal developed AIDS and died after 18 months. Three baboons, inoculated with SIV, developed an immune response against viral proteins including the gp32-34 as measured by Western blot analysis while their sera did not react with PT-1 and PT-2 peptides (data not shown). The infected baboons did not show laboratory or clinical signs of immunosuppression.

All 17 sera tested from African green monkeys were positive in Western blot against SIV proteins but did not react with the synthetic peptides (Table 19). Similarly, Western blot SIV seropositive and seronegative samples from antibodies of three talapoin monkeys and 5 HIV-2 positive human sera did not react with the synthetic peptides (Table 19). The lack of detection of antibodies in these species suggests that the amino acid sequences may be poorly conserved in the cross immunoreactive viruses infecting animals and humans tested. This hypothesis is supported by the finding of a lower degree of amino acid identity found in the human HIV-2 isolates $HIV\text{-}2_{ROD}$, $HIV\text{-}2_{NIH\text{-}Z}$ and $HIV\text{-}2_{ISY}$ (29,30,114) in the region corresponding to the PT-1 and PT-2 peptides of SIV (Figure 40). The termination codon in the SIV envelope is located immediately after the acceptor splice site of the transactivator gene of SIV (28). It is, therefore, possible that messenger RNA independent from the env transmembrane mRNA could be generated

through a splicing mechanism that uses this splice site. To investigate whether a specific protein encoded by this region could be detected, a rabbit antiserum against the synthetic peptide PT-1 was generated. The reactivity of the anti-PT1 serum was tested against the metabolically labelled proteins of SIV and HIV-$2_{NIH-Z}$ infected cell lines in radioimmunoprecipitation (RIP) assay and in Western blot of unlabeled proteins from the same cell lines. While the sera from SIV infected monkeys did recognize specific viral proteins in the RIP assay of $^{35}S$ metabolically labeled protein of the SIV infected KW1 cell line, the anti-PT1 serum failed to immunoprecipitate specific protein(s) (Figure 41, first panel). Similar data were obtained when the RIP assay was performed on HIV-1 and HIV-2 infected cells. The anti-PT1 serum also failed to detect specific protein in a Western blot on cellular lysate of SIV and HIV-2 infected cells (data not shown).

b) BIOLOGICAL ACTIVITY OF RECOMBINANT HIV-$2_{ISY}$

The recombinant phage clone, HIV-$2_{ISY}$ was transfected into the human neoplastic cell line Hut-78. The supernatant of the cell culture was found positive for magnesium dependent reverse transcriptase one week after transfection. Viral expression was confirmed by immunofluorescent straining of the infected cells using HIV-2 positive serum. Genomic DNA was isolated from the infected cells, restricted with endonucleases, electophoresed and blotted according to standard procedures (85,88). Southern blot analysis of the total genomic DNA isolated from the infected cell line indicated the presence of viral sequences (bottom of Figure 27). Hybridization of Xba I and Eco RI cleaved DNA's to the SIV gag gene probe (B16) revealed the same internal bands for the uncloned parental HIV-$2_{SBL6669}$ and the HIV-$2_{ISY}$ proviral DNA, indicating that HIV-$2_{ISY}$ is representative of the majority of the genotype present in the parentally infected cell line. Different restriction enzyme patterns were observed with the genomic DNA isolated from the SIV and the HIV-$2_{NIH-Z}$ infected cell lines. Electron microscopic analysis performed on the HIV-$2_{ISY}$ (HIV-$2_{SBL/ISY}$) transfected cells revealed the presence of mature virions with the expected cylindrical shaped core typical of lentiviruses (Figure 42) and budding particles from the cell membrane (see

inset of Figure 42), indicating that the transfection of the HIV-$2_{ISY}$ DNA induced a productive infection of the Hut-78 cell line.

Immunological Characterization of the HIV-$2_{ISY}$

Western blot analysis and radioimmunoprecipitation were performed on the viral particles obtained from infected Hut-78 cells (Figure 43). The nitrocellulose strips containing unlabeled virion proteins were reacted with a sera from an $SIV_{MAC}$ experimentally infected monkey (Figure 44, lane 1), an HIV-2 infected individual (Figure 44, lane 2) and a normal donor (NS) as well as a mouse monoclonal antibody directed against the HIV-2/$SIV_{MAC}$ major gag protein (p24-26), and control ascites fluid (C). The most reactive and apparently most abundant viral proteins detected in the HIV-$2_{ISY}$ and $SIV_{MAC}$ virions were the gag p24-26 and p15 proteins (see the first two panels of Figure 43). Similar results were obtained when radiolabeled HIV-$2_{ISY}$ virion proteins were used in radioimmunoprecipitation (see left panel of Figure 43). The envelope glycoprotein gp120 was barely detected by immunoprecipitation and not at all by Western blots (Figure 43). The DNA sequence of the replication competent proviral clone lacks a termination codon in the transmembrane portion of the envelope gene and should yield a transmembrane envelope glycoprotein of around 40 Kd. A very faint band located around 40 Kd could be detected in RIP or Western blot assays of HIV-$2_{ISY}$ using positive human sera. However, a well characterized specific antiserum will be needed to clearly define this protein band. A smear, probably representing proteins with different relative migration rates were detected around 30 Kd in $SIV_{MAC}$. This smear has been interpreted as the truncated form of the transmembrane protein (74) although the amino acid sequence after the termination codon is expressed in infected animals (115). A smear could also be detected in the same region in HIV-$2_{ISY}$ using the human serum from a patient infected with HIV-2.

Host Range and Cytopathic Effect of HIV-2

The Hut-78 cell line producing the HIV-$2_{ISY}$ was expanded and virus was concentrated from 10 liters of supernatant as described (95). The HIV-$2_{NIH-Z}$ isolate was used in a parallel experiment to infect the same cell lines. Equal amounts of concentrated virus were then used to infect several human cell lines. Replication and propagation of the virus was monitored by reverse transcriptase assay of the culture supernatant and immunofluorescence on fixed cells. The biological effect exerted by the HIV-2 isolates on the infected cells was measured by counting the number of viable cells and syncytia at different time intervals. The results on the infectivity of the HIV-2 isolates are reported only for the HIV-$2_{ISY}$ isolate (Table 20). HIV-$2_{ISY}$, as well as HIV-$2_{NIH-Z}$ infected the HTLV-I transformed T-cell line MT-2, the T-cell clone 55 immortalized by a single defective copy of HTLV-I (116) and the CEM, Hut-78, Molt-3, H9 and U939 neoplastic cell lines. The highest cytopathic effect, exerted by both HIV-$2_{ISY}$ and HIV-$2_{NIH-Z}$ was observed in the HTLV-I infected cells and in the H9 cells (Table 20), and is coincident with the highest number of syncytia present in the cell culture (Figure 18). The parental virus HIV-$2_{SBL6669}$ also infected Hut-78, U937 clone 16, CEM, and Jurkat T-cells (Table 21), with the highest cytopathic effect observed on the Jurkat and U937-16 cell lines (Table 21).

C) ANIMAL MODEL FOR HIV-2

We described above the isolation of an infectious HIV-2 molecular clone, HIV-$2_{ISY}$ (57). This virus infects both human T cells and macrophages. HIV-$2_{ISY}$ cell-free virions and the HIV-$2_{NIH-Z}$ viral isolate (29) were used to intravenously inoculate Rhesus macaques and African green monkeys. Animal studies were performed by Dr. Genoveffa Franchini of NCI and Dr. Philip Markham of Advanced Bioscience Laboratories, Inc., Kensington, Maryland.

The monkeys were bled monthly and sera were tested for antibody reactivity against HIV-2 antigens by ELISA, Western blot and radioimmunoprecipitation assays. According to Western Blot analysis, seroconversion occurred in animals #172, 176, 177 within the second and third month. One inoculated Rhesus macaque (#173) failed to seroconvert and no antibodies were

found in the inoculated African green monkeys or in the uninfected control animals. The sera of animals that seroconverted recognized the HIV-2 envelope glycoproteins and the major gag protein.

In order to evaluate whether the viral infection was eliciting a constant immune response, the sera of the infected animals obtained at different time intervals was diluted 1:50 to 1:1250 and tested by ELISA. The results indicated that the antibody titer remained constant throughout the year in animals #172 and 177; whereas, animal #176 had a transient increase in antibody titer between 6-8 months post-inoculation (Figure 45). In order to identify which antigens are recognized by the infected animals, radioimmunoprecipitation assays were performed on metabolically labeled proteins obtained from HIV-$2_{NIH-Z}$ infected T cells using sera diluted 1:100 from infected animals. Antibodies directed against envelope proteins (gp160/120) were detected. The antibody titer increased over time. A human serum which was used as a positive control immunoprecipitated the major gag protein (p24) while serum from animal #176 weakly precipitated p24.

Sera from infected animals were tested for the presence of neutralizing antibodies to HIV-$2_{ISY}$ and HIV-$2_{NIH-Z}$ using CEM-SS cells as targets for cell-free virus (67,117). Sera was diluted 1:10 to 1:270 and incubated with the virus for 1 hour at 4°C prior to infection of the target cells. Virus expression was monitored by immunofluorescence assay for HIV-2 p24 using a specific monoclonal antibody (provided by F. Veronese). Only serum from animal #172 showed significant neutralizing antibodies against the homologous virus (Table 22). Since animal #172 was infected with HIV-$2_{NIH-Z}$ and the serum failed to neutralize HIV-$2_{ISY}$, the neutralizing ability of serum obtained from #172 is type specific. The sera of both animals inoculated with HIV-$2_{ISY}$ did not neutralize either of the two viruses.

Immunohematological analysis of the peripheral blood cells obtained from infected animals was performed every other month after infection over a 1 year period at which time the assay was performed monthly. The absolute number of $CD4^+$ T cells in the peripheral blood were monitored because it is one of the most reliable predictive tests of disease development in humans.

Approximately 9 months after infection a decrease in $CD4^+$ T cells was detected in the blood of animals #176 and 177 (Figure 46). This decrease in $CD4^+$ T cells remained constant with cell counts under 1000 $CD4^+$ T cells/$mm^3$. Normal pre-infection values ranged from 1500 to 2000. Although, animal #172 showed fluctuations in the $CD4^+$ T-cell number, a steady decrease of $CD4^+$ cells was not observed. Of the uninfected control animals, #178 showed no decrease or significant fluctuation. Animal #179, which initially had a low level of $CD4^+$ T cells (approximately 1000 cells per $mm^3$), was kept as a control and maintained a constant $CD4^+$ T cell level throughout.

Virus was isolated monthly from the peripheral blood of the infected monkeys. Uninfected Rhesus macaque peripheral blood mononuclear cells or human neoplastic T cell lines were used as targets for virus isolation. Virus was readily recovered during the first 5 months after infection from animal #177. In order to verify that the virus isolated from the infected animal was the virus inoculated, Southern blot analysis was performed on DNA extracted from infected cultured cells. The BamHI digestion profile of the proviral DNAs of the original virus HIV-$2_{ISY}$ as well as those for the 1 and 5 month isolates obtained from animal #177 were indistinguishable by hybridization with the three internal BamHI fragments of HIV-$2_{ISY}$ clone.

This experimental system provides a unique model to study genetic variation since the virus inoculum was obtained from a molecularly cloned provirus which represents a single genotype. In order to determine the degree of genetic variation occurring in vivo in the viral genome, molecular clones of the provirus after 1 and 5 month passage of the virus in animals were constructed. The provirus was cloned within 1 1/2 months of cell culture in order to minimize the time spent in culture and possible mutations that can occur with in vitro passage. We determined the nucleotide sequence of the entire envelope gene of both proviruses. Comparison of the DNA sequences of the virus recovered 1 and 5 months post infection (HIV-$2_{ISY-1}$, HIV-$2_{ISY-5}$, respectively) with that of the molecularly cloned virus used in the inoculation indicated that few changes in nucleotide sequence had occurred during infection in monkeys (Figure 47). In HIV-$2_{ISY-1}$ a single nucleotide change was detected in the third codon position (C-> A) which did not change the encoded amino acid (proline) located in the extracellular portion of the envelope protein (gp120) at position 238.

Five nucleotide changes were detected in the HIV-$2_{ISY-5}$ clone. Three of these changes did not result in a change of the encoded amino acid at positions 103, 232, and 454 (Figure 47). Two changes resulted in a change from a negatively charged amino acid (glutamic acid) to a positively charged amino acid (lysine) at positions 414 and 745. The single nucleotide change detected in the HIV-$2_{ISY-1}$ env gene was not present in the HIV-$2_{ISY-5}$ env gene.

## 4. Discussion

Genetic Analysis of SIV and HIV-2 Genomes

The identification of an increasing number of human and simian retroviruses in the last seven years makes it imperative to determine the precise genetic relationship of these viruses in order to elucidate the genetic basis for their pathogenic effects. Studies on the replicative functions of HIV-1 and its role in T-cell killing have shown that these human retroviruses have a more complicated mechanism of regulation than the non-primate retroviruses. The discovery of a second group of viruses in both primates and humans which are structurally and genetically related to HIV-1 and are also associated with immunodeficiency calls for a reinterpretation of the natural history of these viruses and for a reevaluation of the hypothesis that AIDS is a new disease. In fact, analyses of the rate of nucleotide changes suggests that HIV-1 and HIV-2 might have diverged from each other as recently as 40 years ago (G. Meyers, Los Alamos) while the first documented cases of AIDS or aggressive form of Kaposi in young people date as far back as the early 1960s (118).

The molecular characteristics of SIV and HIV-2 reveal that their genetic organization is very similar to HIV-1. SIV and HIV-2 are more closely related to each other than to HIV-1. Nevertheless, the extent of similarity among these primate viruses indicate that they arose from a common ancestor. The immune cross reactivity of $SIV_{MAC}$, HIV-1 and HIV-$2_{ROD}$ in the major core proteins reflects the high conservation of the gag gene sequence. Comparison of the gag protein of $SIV_{MAC}$, HIV-1 and HIV-2 with other members of the lentiviridae family demonstrate that the former represents a group of viruses that must have diverged from each other more recently

than they diverged from the latter group and that SIV and HIV-2 diverged from each other more recently than either diverged from HIV-1.

The vif gene of $SIV_{MAC}$ as well as of HIV-2 appears to be less conserved than all the other genes. The biological significance of this observation is not clear.

Conversely, the high degree of conservation of the central region of the nef protein between these three viruses suggests that this region may be the functional domain of the protein. The 27 Kd HIV-1 nef protein is a cytoplasmic protein (119) that is not required for viral replication in vitro (13). The conservation of the amino acid sequence of the nef proteins would suggest its importance in the biology of these primate retroviruses, perhaps as a negative regulatory element.

One point of considerable debate is whether the West African viruses (HIV-2) cause AIDS in people. Early reports, which identified a human virus related to SIV, on the basis of serology, suggested a lack of disease association (120) while others reported the isolation of HIV-2 viruses from a few patients with immunodeficiency and no signs of infection with HIV-1 (69). More recently, an increasing number of HIV-2 isolates have been obtained from patients with AIDS from West Africa (70,74). However, a retrospective seroepidemiological study on 4,248 people in West Africa showed the absence of any clinical signs in 330 infected people (121). The changes in the envelope proteins of two HIV-2 isolates are similar which suggests that HIV-1 and HIV-2 have existed in their present population for similar lengths of time. Therefore, it is possible that the discrepancy between these studies is due to a lower morbidity rate for HIV-2. A more difficult question is whether there is a fundamental genetic difference between the two virus groups that could explain their apparent different biological behavior in infected individuals. The overall genetic structure of HIV-1 and HIV-2 is very similar, with the exception of an extra open reading frame in HIV-2, which has been designated vpx.

Most of the viral gene products that regulate viral expression and replication of HIV-1 are also present in HIV-2. In fact, the putative functional domains of the regulatory proteins are evolutionary conserved. However, differences in the overall structure of the HIV-2 LTR's which are larger than HIV-1 LTR's account for a variation in the responsive region to the viral

transactivator gene (tat) (122). More genetic information is also needed to encode the tat and rev proteins as reflected by the study of functionally active SIV and HIV-2 cDNA's (101). However, the major structural differences appear to be the presence of a gene (vpu) in type 1 viruses (28) and another gene (vpx) which is present only in type 2 viruses (83,98). The amino acid sequences of these two genes are not homologous and whether they are functionally equivalent is still an open question.

$SIV_{MAC}$ and HIV-2 viruses also differ from HIV-1 in the length of the transmembrane portion of the viral envelope. Whereas HIV-1 encodes for a gp41, $SIV_{MAC}$ contain a translation termination codon reducing the transmembrane protein to 32 Kd (gp32). In addition, some isolates of HIV-2 also appear to have a truncated transmembrane protein (25,29,30,123). This work shows that the region after this termination codon is expressed and immunogenic in SIV infected monkeys. Two out of three experimentally infected macaques showed reactivity against one or both synthetic peptides, PT-1 and PT-2. No definitive correlation between reactivity against PT-1 and PT-2 and disease progression could be found since all three macaques died of AIDS from 17 to 18 months following infection. It is intriguing that three SIV inoculated baboons did not mount an immune response to the generated peptides. These baboons were SIV virus positive and antibody positive although they remained healthy 2 1/2 years after inoculation. This may suggest an in vivo modulation of expression of the termination codon that is correlated with pathogenic effect.

No reactivity against PT-1 was detected in the other SIV-infected animals studied or in HIV-2 infected humans. As genetic variability occurs among the various isolates of HIV-1 and HIV-2, it is likely that different monkey species are infected with related SIV viruses with slightly different amino acid sequences in this region. This assumption is underscored by the fact that the region in the transmembrane protein after the termination codon is one of the least conserved regions between SIV and different HIV-2 isolates (83,98,122). Likewise, the lack of immune reactivity in HIV-2 infected humans may well be the result of a different amino acid sequence in the region of HIV-2 homologous to the region in SIV from which PT-1 and PT-2 were derived.

The availability of a replication competent HIV-2 proviral clone provides the tools to study the vpx gene which is unique to HIV-2 and SIV as well as the role of the "non-essential" accessory genes of HIV in virus replication and the relevance of the structural differences in vivo. Furthermore, the regulatory elements of the viral LTRs and their interaction with the regulatory proteins can be evaluated in the context of a complete infectious genome. The value of an infectious HIV-2 clone in developing an animal model for HIV vaccine and therapy studies should also be emphasized. Rapid progress in the development of a protective vaccine against HIV has been impaired by the lack of a suitable and cost effective animal model. Successful infection of non-human primates has been achieved only in chimpanzees (124) and gibbons (125) which are scarce. Furthermore, no pathogenicity of the virus is observed in these animals. Since the parental HIV-$2_{SBL6669}$ and the molecular clone HIV-$2_{ISY}$ productively infect rhesus macaques and these macaques are sensitive to the pathogenic effect of HIV-2, the development of an animal model using a highly related human virus will obviously be very valuable. Finally, we have recently shown that individual isolates of HIV-1 are composed of microvariants with distinct biological properties and susceptibility to given neutralizing sera (126). The composition of this population presumably drifts due to new mutations and selection in response to changes in available target cells and host immunity. The availability of an infectious molecular clone will allow us to measure the genetic evolution of the viral genome and its immunological consequences in the infected host.

Animal Model for HIV-2

Minimal genetic drift of the HIV-2 viral genome was found to occur in vivo over the course of a 5-month period in the macaques infected with a cloned virus isolate. Such data suggest that the high degree of HIV-1 genetic variability first described by Hahn et al. (127) and more recently reexamined by polymerase chain reaction (128) might be due to selection of viral genotypes present within the infected individuals rather than to rapid nucleotide changes in the viral RNA. This concept is supported by the demonstration that each viral strain contains multiple genotypes (126) and implies that HIV-2 and HIV-1 existed in myriad variants at the start of the epidemic. An

alternative explanation of the low genetic drift detected in the HIV-$2_{ISY}$ isolated from Rhesus macaques could be that the virus is not subjected to selective pressure due to the fact that our animals did not develop neutralizing antibodies. However, this conclusion does not take into account the role of cell mediated immunity. The failure to isolate virus from the peripheral blood lymphocytes (PBL) of HIV-2 inoculated Rhesus macaques several months after inoculation seems to suggest a low rate of virus replication in peripheral blood mononuclear cells. However, despite the failure to isolate virus from the PBL of the infected animals, viral sequences could be detected in the PBL DNA of the infected animals by PCR. Since the antibody titer in the animals remained constant, a certain level of viral replication must occur in PBL and in other lymphoid organs. The progressive decrease of $CD4^+$ T cells from the peripheral blood of the two animals infected with HIV-$2_{ISY}$ indicates that a pathogenetic effect is generated even though there is a low level of virus replication. The early signs of immunodeficiency observed in the infected Rhesus macaques suggest that this animal model can be used to study the prevention of infection and the modulation of disease progression. Moreover, the availability of monkeys infected with a molecular clone provides a suitable tool to study genetic manipulation of accessory genes in viral infectivity and pathogenicity in vivo.

These studies have shown that extensive homology exists among the HIV-1, HIV-2, and SIV genomes. Such a similarity in genomic structure and biological properties of these retroviruses suggests that an SIV animal model system can be used to study the mechanism of immunodeficiency disease. Infectious molecular clones of HIV-2 and SIV (129,130) have been used to inoculate rhesus monkeys in order to follow the viral etiology of AIDS within a suitable time frame. The molecularly cloned SIV virus induced SAIDS and resulted in the death of the animals. Animal model studies should lead to an understanding of the molecular mechanisms underlying disease pathogenesis and the effects of drug therapy or other treatments on the development of AIDS.

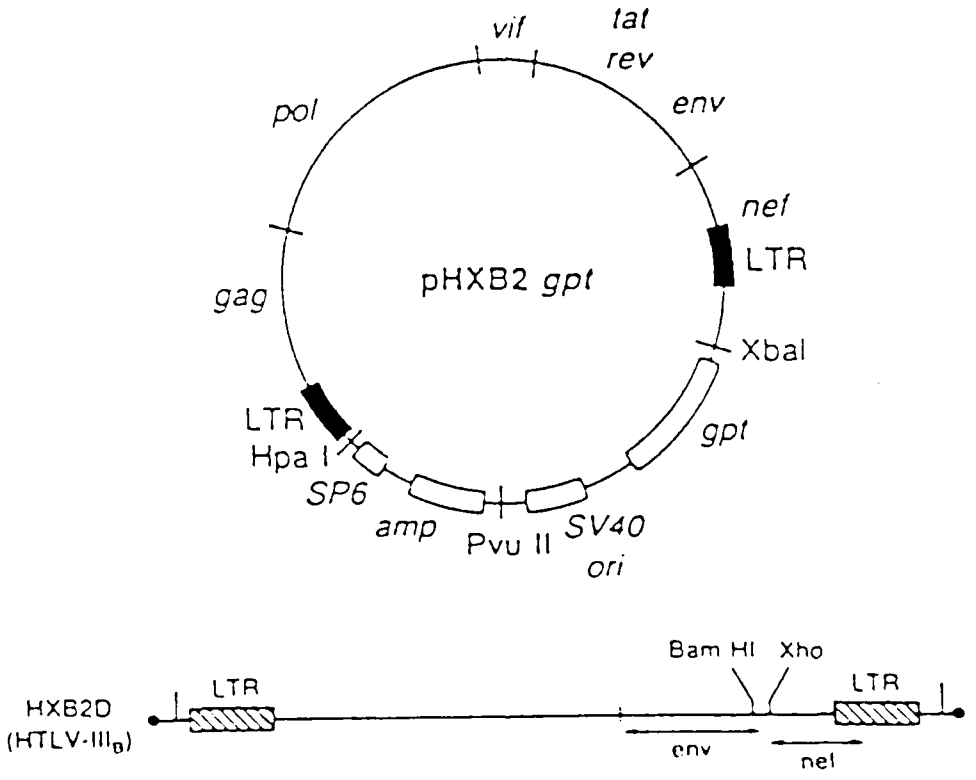
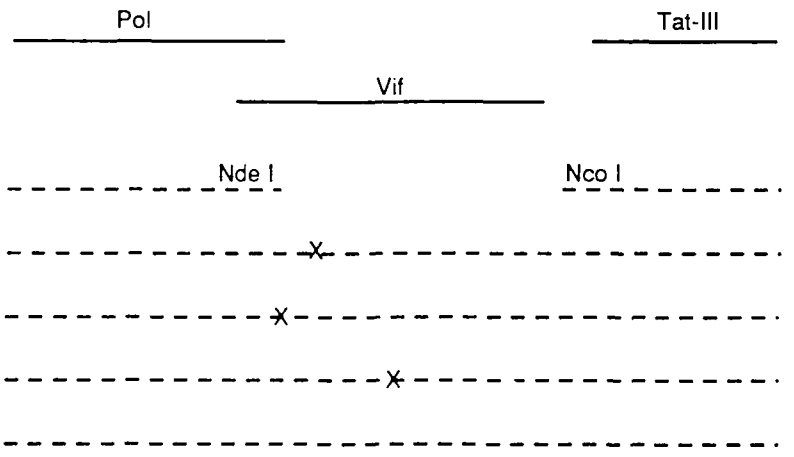



**FIGURE 1: Construction of C-Terminal env Deletion Mutants.**
The parental plasmid pHXB2gpt, which contains a full-length biologically active HIV-1 proviral clone, was cleaved in the 5'-nef region using XhoI. Following variable bidirectional digestion with Bal 31 nuclease, the clones were blunt ended with T4 DNA polymerase and self-ligated with T4 DNA ligase. (The XbaI site is no longer present in the modified clones due to the presence of a polylinker.) Deleted regions were identified by sequencing the clones by the Sanger method using double-stranded DNA and Klenow fragments.




| Mutants derived from pHXB2gpt | Stable infections established after transfection of H9 cells |
|---|---|
| ΔS | 0/6 |
| 6.9 | 0/6 |
| 3.3 | 0/7 |
| 153.0 | 0/3 |
| X | 4/4 |

**FIGURE 2: Construction and Properties of <u>vif</u> Mutants of HIV-1.**
Plasmid X was generated from pHXB2gpt by removal of an EcoRI site in the polylinker region. Mutant ΔS was prepared by digestion with NdeI and NcoI and religation. Mutants 6.9, 3.3, and 153.0 were generated by site directed mutagenesis such that stop codons were introduced in frame at the locations marked ---X--- (see text). Mutated fragments were recloned into clone X and transfected into H9 cells by protoplast fusion. Cultures were monitored for HIV-1 production throughout the course of the experiment.

## AMINO ACID SEQUENCES OF C-TERMINAL GP41 DELETION MUTANTS

```
781
HXB2  ivellgrrgwealkywwnllqywsqelknsavsllnataiavaegtdrvievvqgacra
X10.1 ..............................................................
X9.3  ..............................................................
X295  ..............................................................
X362  ..........................................shtsqtfktndlqgscrs---
X429  ..............................................................
X468  ..........................................------------------
X269  ..............................................................r

      841
HXB2  rhiprrirqglerill
X10.1 .......rrrrwvfqshlryl
X93   ...........aatnaacawleaqeeeevgfpvtpqvplrpmtykaavdlshflkekggl
X295  rhrs---------
X362  ----------
X429  rpsld--------
x468  ----------
x269  s----------

      901
X9.3  eglihsqrrqdildlwiyhtqgyfpd+
```

**FIGURE 3: Amino Acid Sequences of C-Terminal <u>env</u> Deletion Mutants.**
Amino acid sequences of parental HXB2 and selected deletion mutants are shown. Dots indicate residues identical with the parental HXB2 clone, and dashes indicate deleted amino acids. Note the cystine residue at position 835 in clone X362 and the proline residue at position 842 in clone X429.

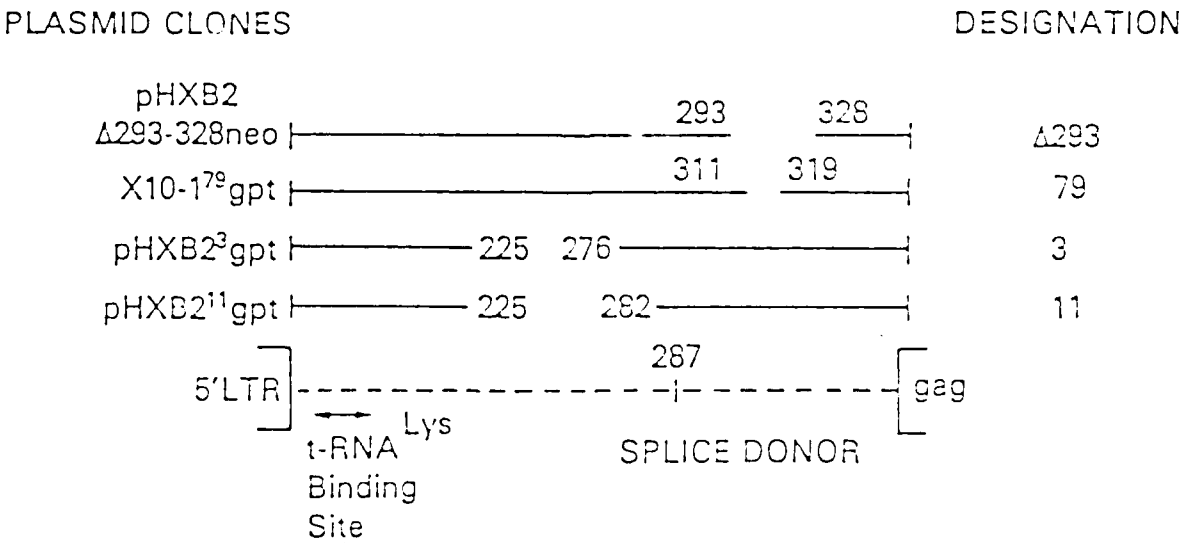



FIGURE 4: Locations of Deletions in Mutants Examined for Defects in Packaging.

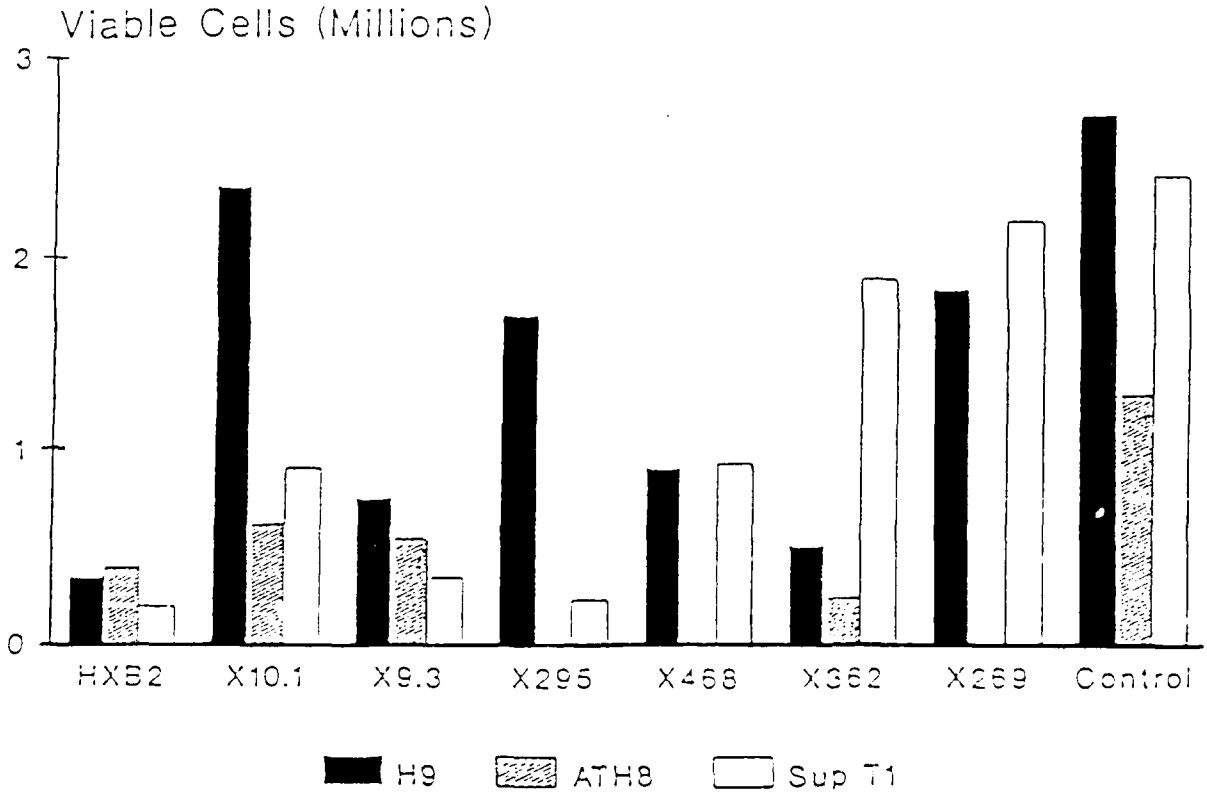



**FIGURE 5: Cytopathic Effects of Cell-Free Transmission of C-Terminal <u>env</u> Deletion Mutants.**
Numbers of viable cells surviving after infection with 300 x TCID50 (H9 and SupT1) or a fixed multiplicity of infection (1000 cell-1 - ATH8) of the respective deletion mutants after two weeks incubation. Experiments on H9 and SupT1 were performed in triplicate, those on ATH8 in duplicate. Cytopathogenicity determinations on ATH8 were not performed on X295 and X468, whereas X269 was found to be extremely cytopathic (no viable cells seen).

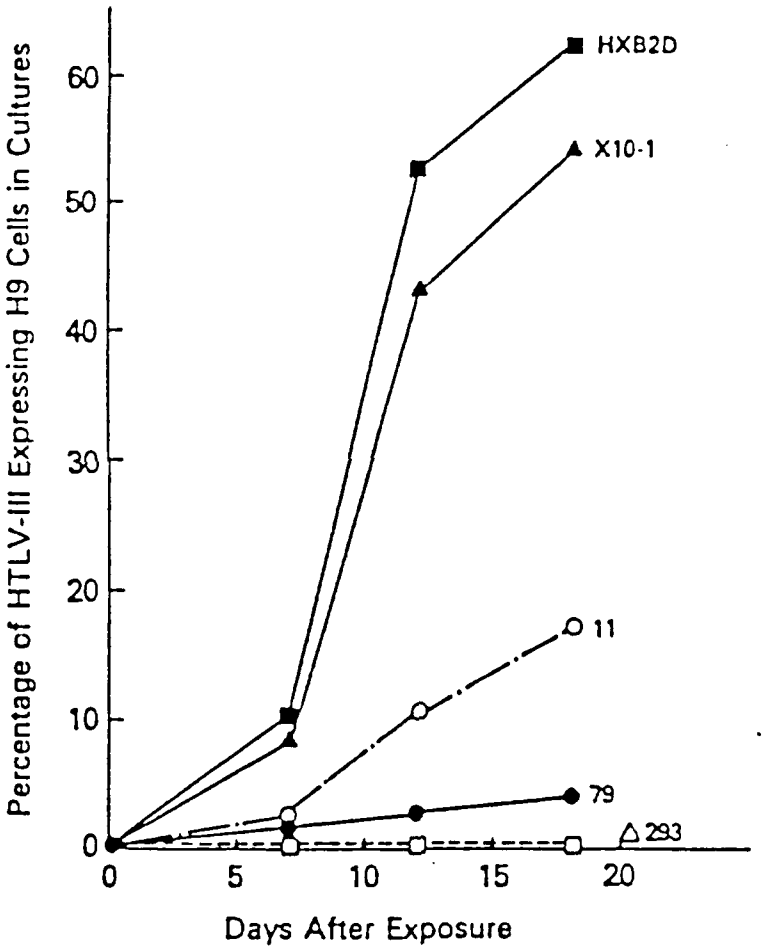



**FIGURE 6: Transmissibility of "Packaging" Mutant Viruses into H9 Cells.**
Plasmid DNA containing the deletions in the sequences between the 5'LTR and gag (Δ293, #79, and #11) were transfected into Cos-1 cells. The resulting supernatants were tested for the presence of infectious virus by cocultivating with polybrene treated H9 cells and monitoring the percentage of cells positive for HIV-1 p24 by immunofluorescence. Plasmids HXB2D and X10-1 were included as positive controls.

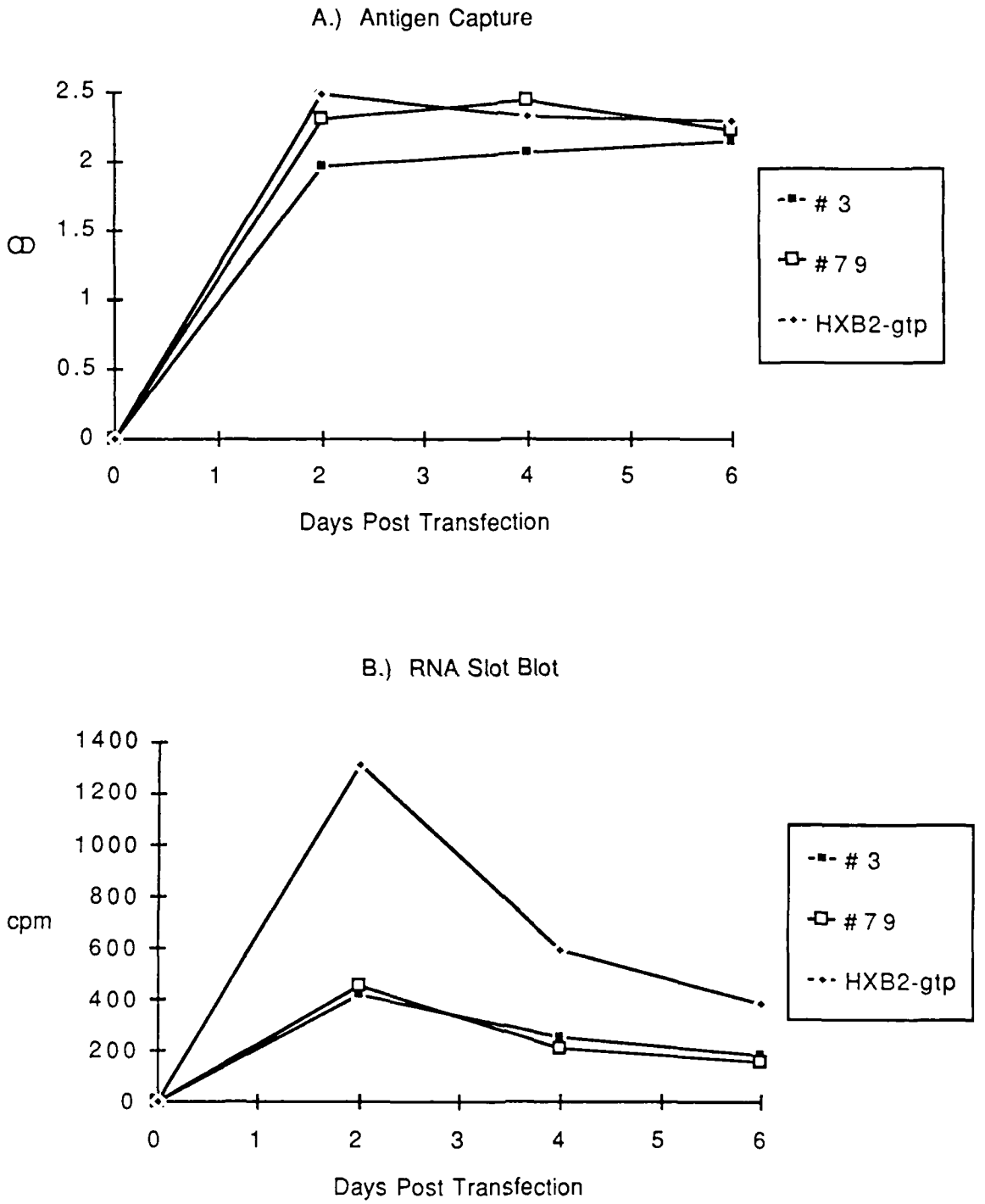



**FIGURE 7. Time Course of the Amount of HIV-1 p24 and HIV-1 RNA Accumulating in Extracellular Virions Following Transfection of Plasmid DNAs into COS-1 Cells.** Cos-1 cells were transfected with wild type (HXB2 gtp) or mutant (#3, #79) DNA, and the kinetics of appearance of viral p24 antigen and RNA in the cell-free supernatants was measured at various times post infection. **(A)** HIV-1 p24 was quantitated by Biotech's antigen capture assay. **(B)** HIV-1 RNA was quantitated by slot blot assay and hybridization with $^{32}$P HXB2.

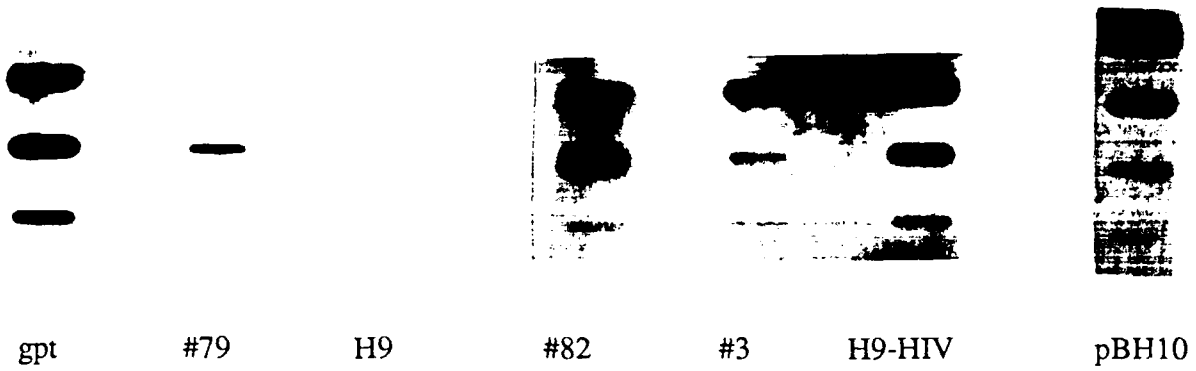



**FIGURE 8: Slot Blot Hybridization of 5-Fold Serial Dilutions of RNA Extracted from Virions and Hybridized with $^{32}P$ pBH10 Probe.**
The last two sets include positive controls of dilutions of RNA from HIV grown in H9 cells and the plasmid pBH10. Note that a "mock" virus preparation from uninfected H9 cells gives no hybridization signal.

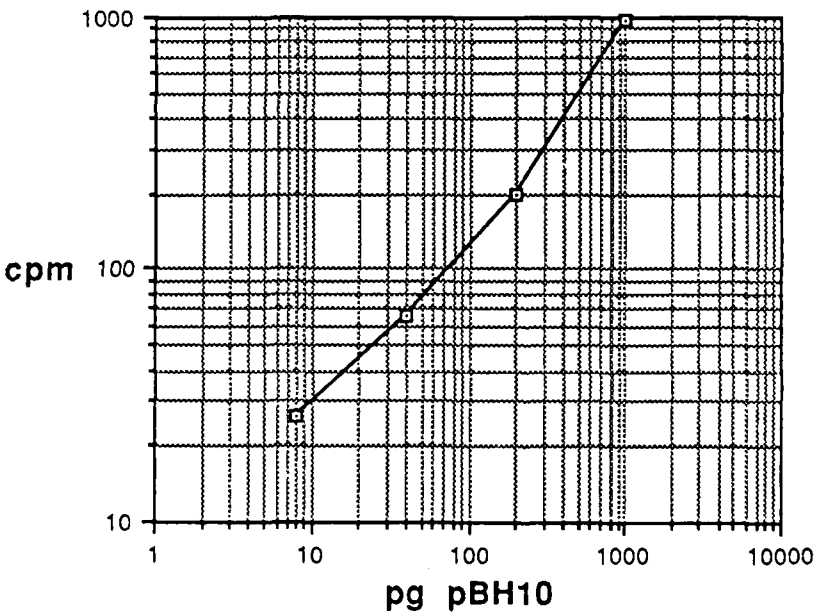



**FIGURE 9: Standard Curve of cpm Versus pg pBH10 from Slot Blot Data.**
Serial dilutions of pBH10 were slot blotted on nitrocellulose filters and hybridized with $^{32}P$ labeled pBH10 probes. The region of the filter containing the hybrids was cut out and counted in a scintillation counter. The cpm hybridized was plotted against the amount of pBH10 target applied to each slot.

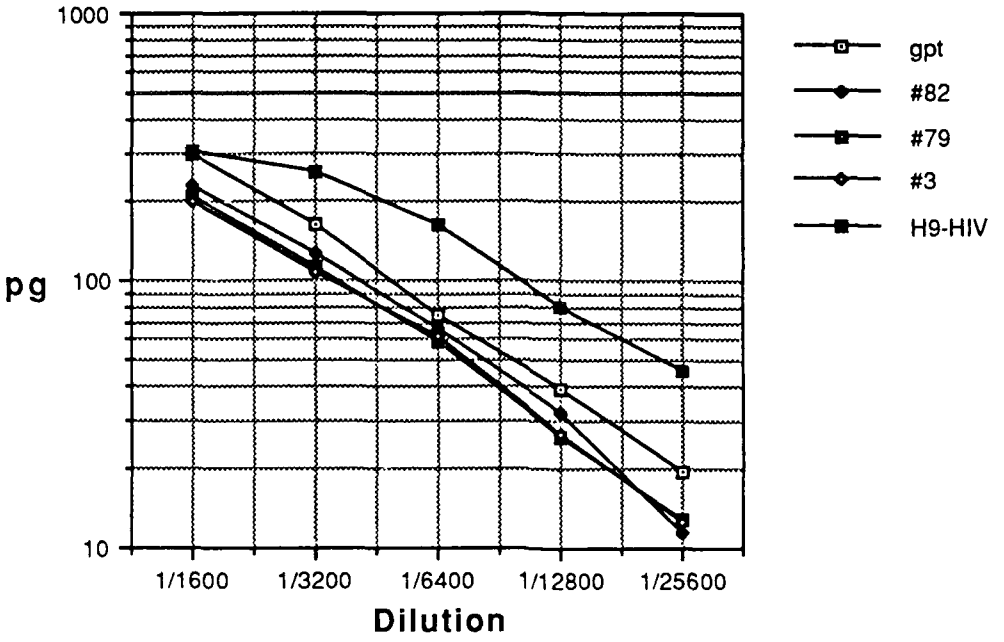



**FIGURE 10: Quantitation of Viral Antigen in Cell-Free Virions Obtained from "Packaging" Mutant Viruses.**

Serial dilutions of cell-free virions were assayed for p24 activity using Biotech's p24 Antigen Capture assay. The results are plotted as pg antigen present at each dilution. The linear portion of the curve was used for quantitation purposes.

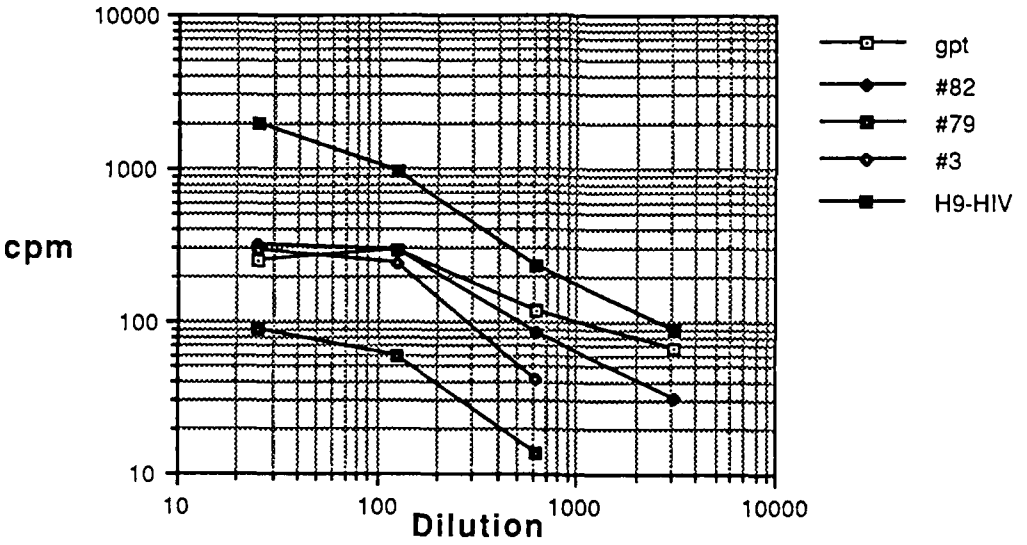



**FIGURE 11: Quantitation of Viral RNA (cpm) Present in Cell-Free Virions Obtained from the "Packaging" Mutant Viruses.**

Serial dilutions of cell-free virions were assayed for HIV-1 RNA by slot blot hybridization with $^{32}P$ labeled pBH10 probe as in Figure 8. The results are plotted as cpm hybridized at each dilution.

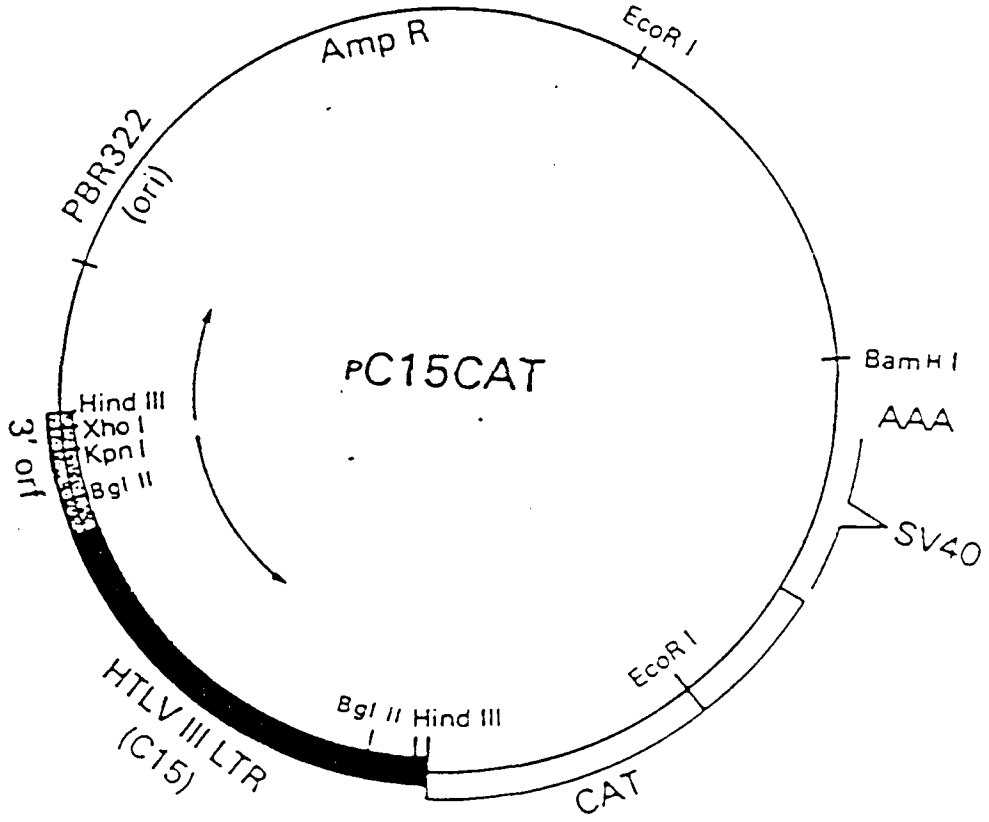
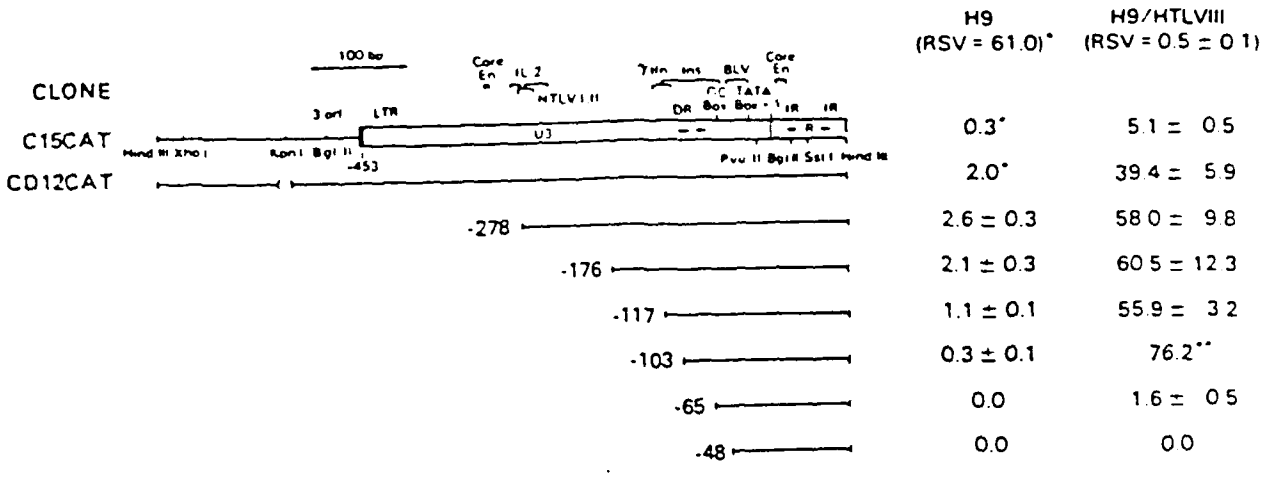



**FIGURE 12: Plasmid Clone C15CAT.**
Plasmid Clone C15CAT was constructed by ligation of Hind III linkers to the blunt ended Pst I insert of C15, digestion with Hind III and ligation of the resulting fragment into the Hind III site of pSVOCAT. Deletion mutants in LTR were made by Bal31 exonuclease digestion from the KpnI site in the direction of arrows.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ATG | GAG | CCA | GTA | GAT | CCT | AGA | CTA | GAG | CCC | TGG | AAG | CAT |
| met | glu | pro | val | asp | pro | arg | leu | glu | pro | trp | lys | his |
| — | gly GGG | — | — | gly GGT | — | — | — | gly GGG | — | — | — | — |
| — | gly GGG | — | — | — | — | — | — | — | — | — | — | — |
| — | — | — | — | gly GGT | — | — | — | — | — | — | — | — |
| — | — | — | — | — | — | — | — | gly GGG | — | — | — | — |
| — | — | — | — | ala GCT | — | — | — | — | — | — | — | — |
| — | — | — | — | lys AAA | — | — | — | — | — | — | — | — |
| — | — | — | — | glu GAG | — | — | — | — | — | — | — | — |
| — | — | — | — | — | — | — | — | ala GCT | — | — | — | — |
| — | — | — | — | — | — | — | — | asp GAT | — | — | — | — |

**FIGURE 13: Missense Mutants Generated at the Amino Terminus of tat.**
The relevant nucleotide sequence of the wild-type tat cDNA clone is shown and the alterations made to generate the missense mutations are indicated. Mutant cDNA clones are designated by the position of the amino acid altered and the resulting amino acid.




```
3' orf--->                          Xho I
CCAGCAGCAC ATGCGGTGCG AGCACTATCT CGACACCTAC AAAAACATGC ACCAATCACA  8505

ACTAGCAACA CAGCAGCTAC CAATGCCGCT TGTCCTTCGC TACAAGCACA ACAGCAGCAC  8565
                                  Kpn I
CACGTGGGTT TTCCAGTCAC ACGTCAGGTA CCTTTAACCC CAATCACTTA CAAGGCAGCT  8625
  Bgl II              Polypurine Tract  |(-453)U3--->
GTAGATCTTA GCCACTTTTT AAAAGAAAAC GGGGGACTGG AAGGGCTAAT TCACTCCCAA  8685

CGAAGACAAG ATATCCTTGA TCTGTGGATC TACCACACAC AAGGCTACTT CCCTGATTGG  8745
                                                    Core En
CAGAACTAGA CACCAGGGCC AGGAGTCAGA TATCCACTGA CCTTTGGATC CTGCTACAAG  8805
       IL-2                HTLV I,II |-278  IL-2
GTACTACCAG TTGAGCCAGA TAAGGTAGAA GAGGCCAACA AAGGAGAGAA CACCAGCTTG  8865

TTACACCCTG TGAGCCTGCA TGGAATGGAT GACCCGGAGA GAGAACTCTT AGACTCGAGG  8925
          |-176
TTTGACAGCC GCCTAGCATT TCATCACATC GCCCGAGAGC TGCATCCGGA GTACTTCAAG  8985
<--3' orf       INS
 -1F  ***     |-117          |-103 Direct Repeats
AACTGCTGAC ATCGAGCTTT CTACAAGCGA GTTTCGCCTC CGCACTTTCC AGCGAGGCCT  9045
                                                    TATA
     |-65                  |-48                    Box   Pvu II
GCCCTCGCCC GCACTCGCGA GTCGCCGAGC CTCAGATGCT GCATATAAGC AGCTGCTTTT  9105
                    Core En
    <---U3 |(+1)R--->  IR       Bgl II          Sst I IR
TGCCTCTACT CGCTCTCTCT CGTTAGACCA GATCTGAGCC TGCGAGCTCT CTGCCTAACT  9165
                        Poly A Sig.
                              Hind III              | poly A
AAGGAACCCA CTGCTTAAGC CTCAATAAAG CTTgccttga gtgcttcc               9213
```

**FIGURE 14: Features of Clone C15 and Resulting 5' Deletion Clones Containing HTLV-III-LTR Sequences.**

CD12 contained a small deletion around the KpnI site by restriction enzyme analysis while retaining the surrounding XhoI and BgIII sites. Each in nucleotides from the transcriptional start site at +1. Regions of homology to other genes and biologically relevant features are diagrammed as indicated above the map. CAT assay results of lysates prepared from transfected H9 and H9/III cells are shown on the right of each clone. Balues are percent conversion of $^{14}C$ chloramphenical to acetylated metabolites.

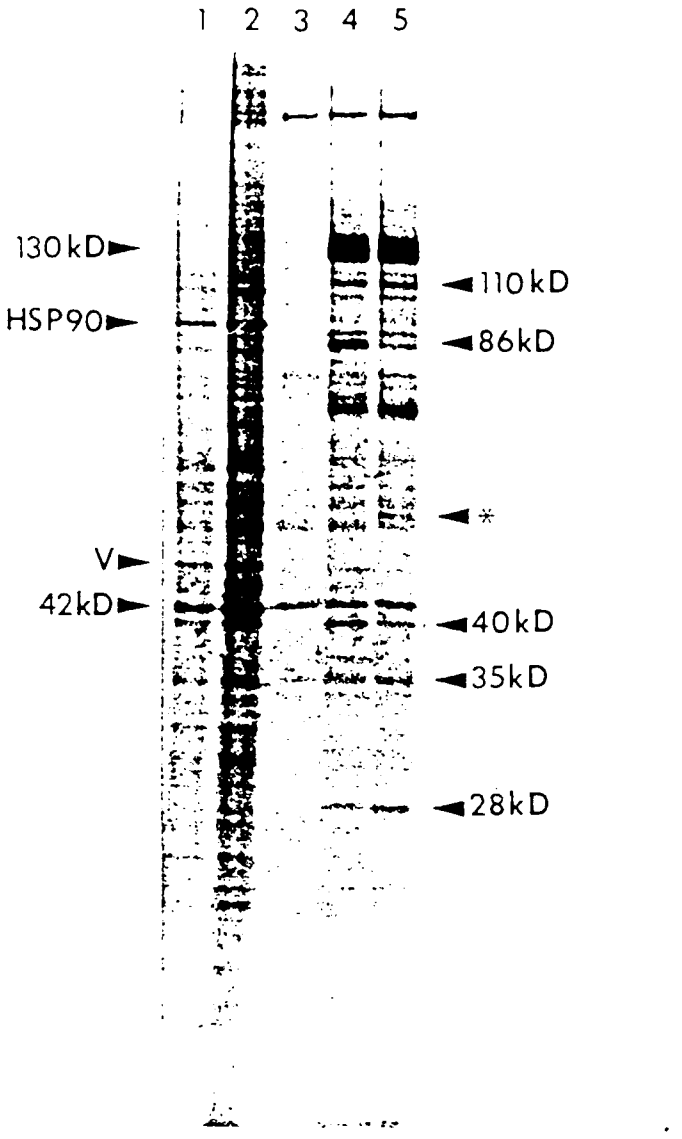



**FIGURE 15: Microscale Affinity Assay of Proteins Binding to the HIV Enhancer Sequence.**

Nuclear extracts of cells labeled with $^{35}$S-methionine were prepared and incubated with biotinylated oligonucleotides containing the enhancer sequence. The protein/oligonucleotide complexes were captured on streptavidin beads. The specific binding proteins were eluted and analyzed on polyacrylamide gels. Lanes 1 and 2 are two different amounts (1/1000 and 1/200, respectively) of the crude nuclear extract used for each reaction. Lane 3 represents the proteins that are recovered when all the ingredients of the reaction except biotinylated probe are added. Lane 4 represents the proteins recovered with the biotinylated HIVEN3c1/2 oligonucleotide, and Lane 5 represents the proteins recovered with the biotinylated HIVEN2m3/4 oligonucleotide. Exposure was for 2 days on Kodak XAR film.

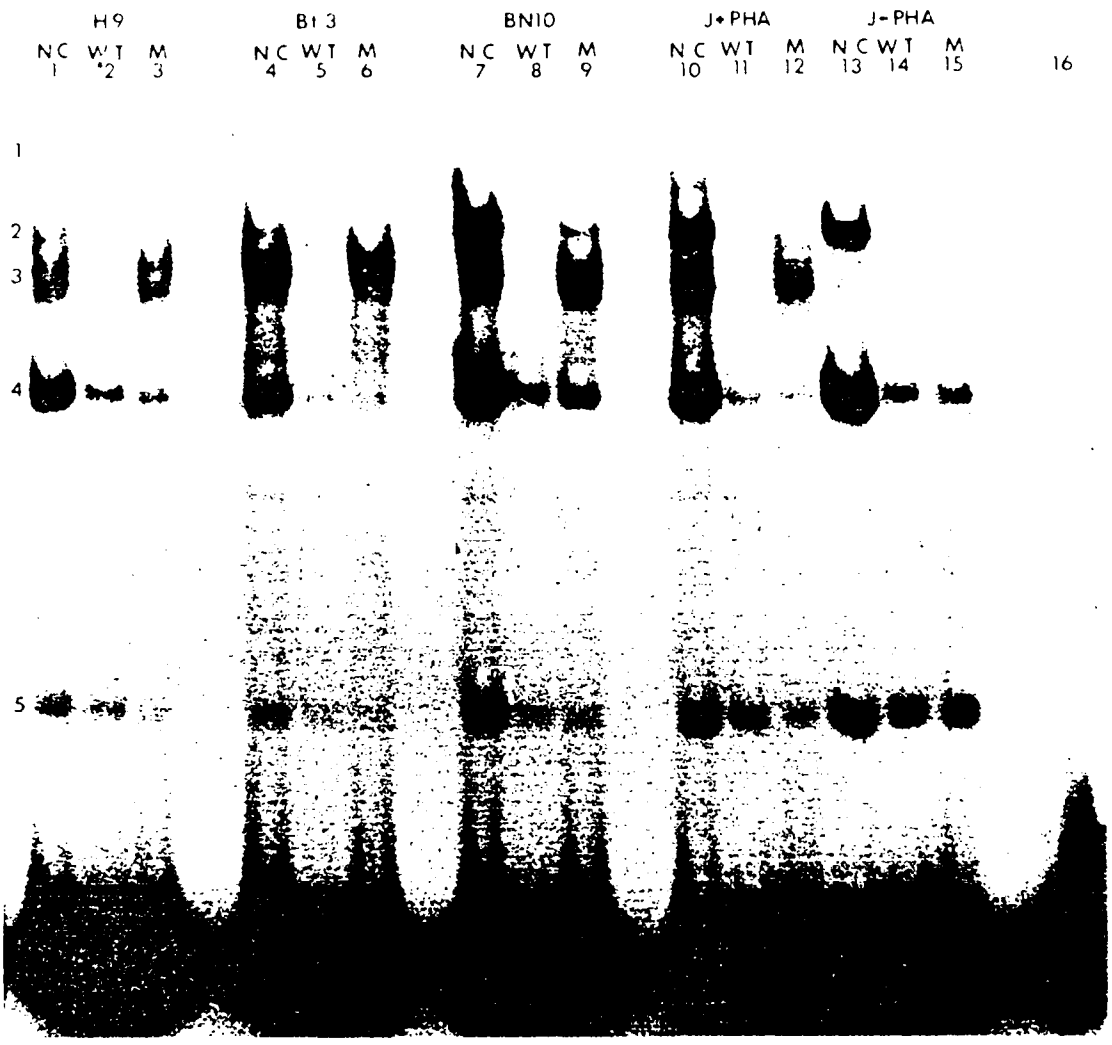



**FIGURE 16: Gel Retardation Assay Using HIV Enhancer Probe.**
Cell extracts were prepared from H9, SK-CML8-Bt3, SK-CML-BN10, PHA-induced Jurkat and uninduced Jurkat cells and incubated with a biotin labeled HIV enhancer 12 bp probe (-GGGACTTTCCAG-). Lanes 2,5,8,11 and 14 also contained 10 pmol HIVEN c 1/2 oligonucleotide competitor. Lanes 3,6,9,12 and 15 contained 10 pmol HIVEN c 3/4 competitor. Lane 16 contained the probe with no added extract.

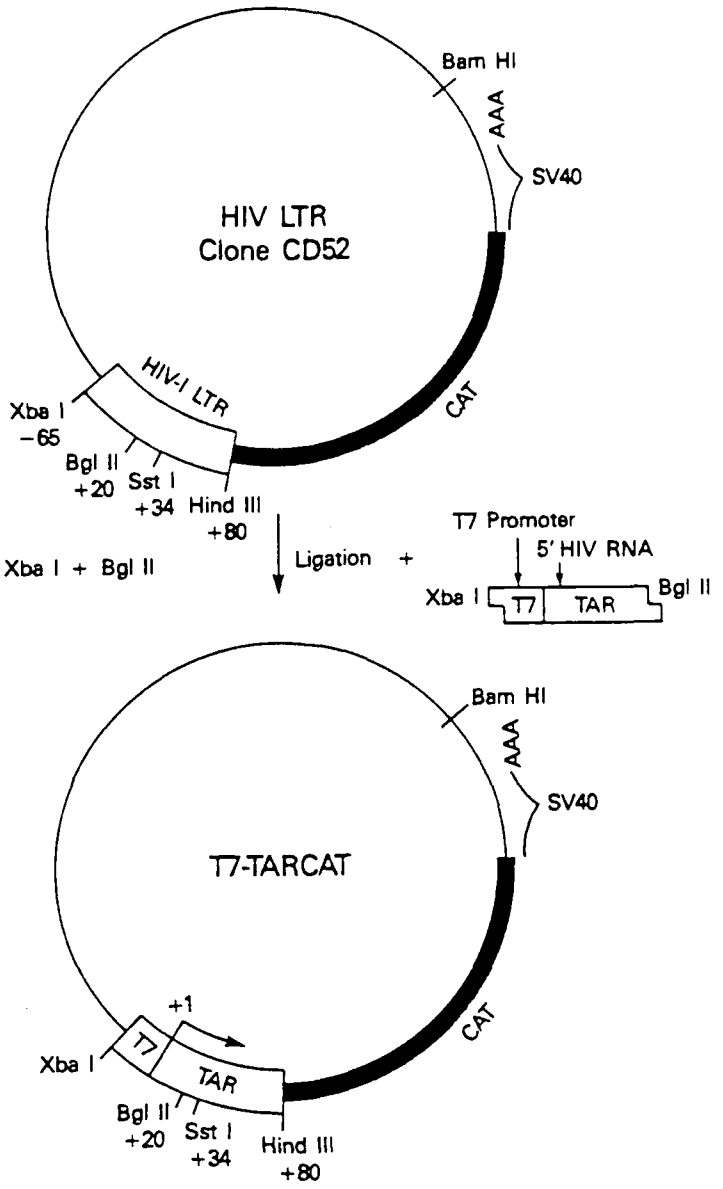



**FIGURE 17: Construction of Plasmid T7-TAR-CAT.**
Plasmids T7-TAR-CAT was constructed from clone CD52 which contained the HIV-1 LTR gene linked to the Chloramphenical amino transferase gene (CAT) and the polyadenylation site of SV40. The T7 promoter was inserted upstream of the TAR region by digestion with Xba I and Bgl II and ligation of a Xba I/Bgl II fragment containing the T7 promoter linked to the 5' region of HIV.

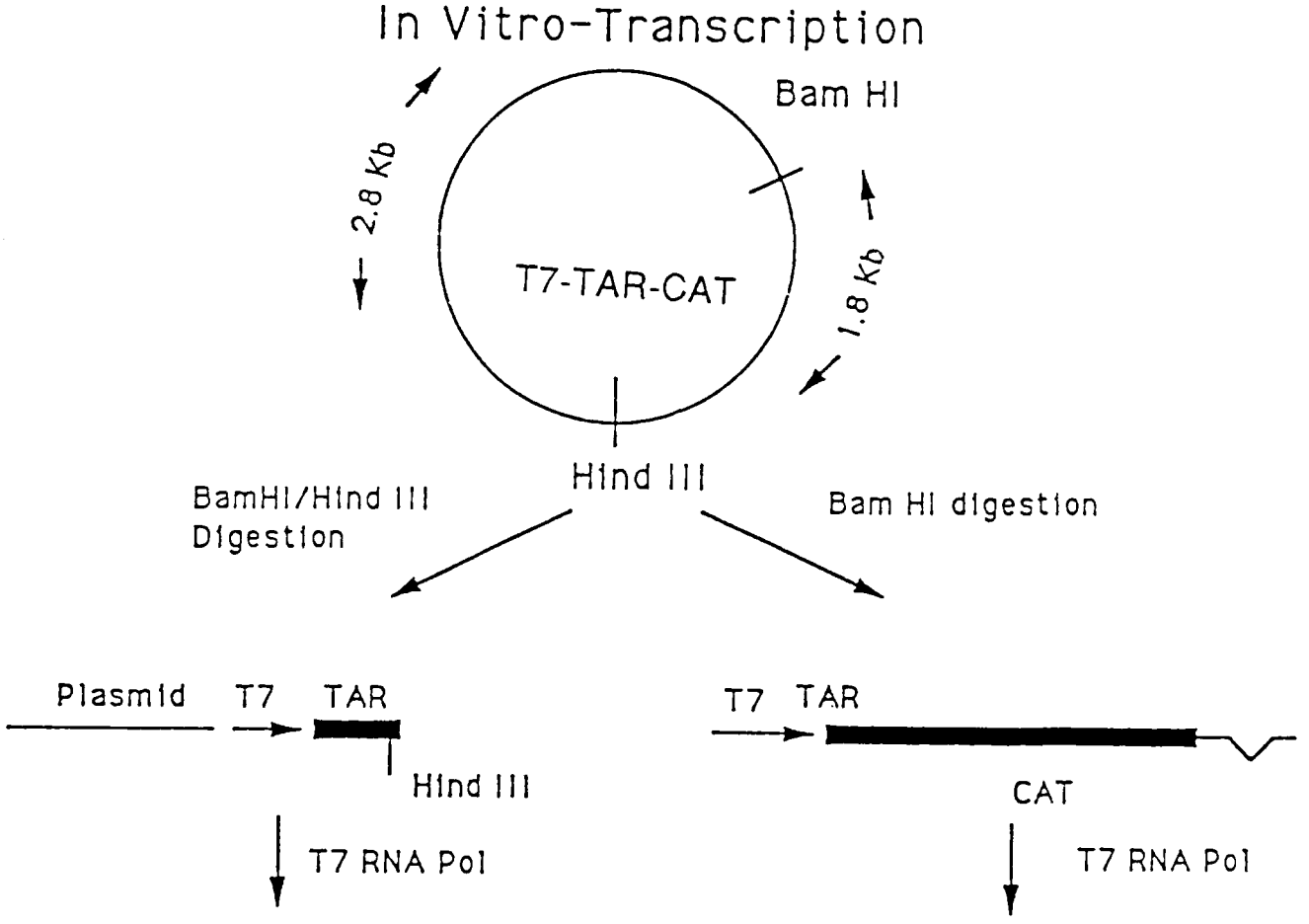



**FIGURE 18: Generation of HIV-1 TAR Transcripts Linked to the CAT Gene.** The T7-TAR-CAT plasmid was linearized by digestion with Bam HI. The resulting DNA was used as a template for T7 RNA polymerase to transcribe mRNA containing the CAT gene linked to the TAR sequences. For generation of transcripts of TAR only, the plasmid was digested with Bam HI and Hind III, the 2-8 kb fragment was isolated on agarose gels, and used as the template for transcription.

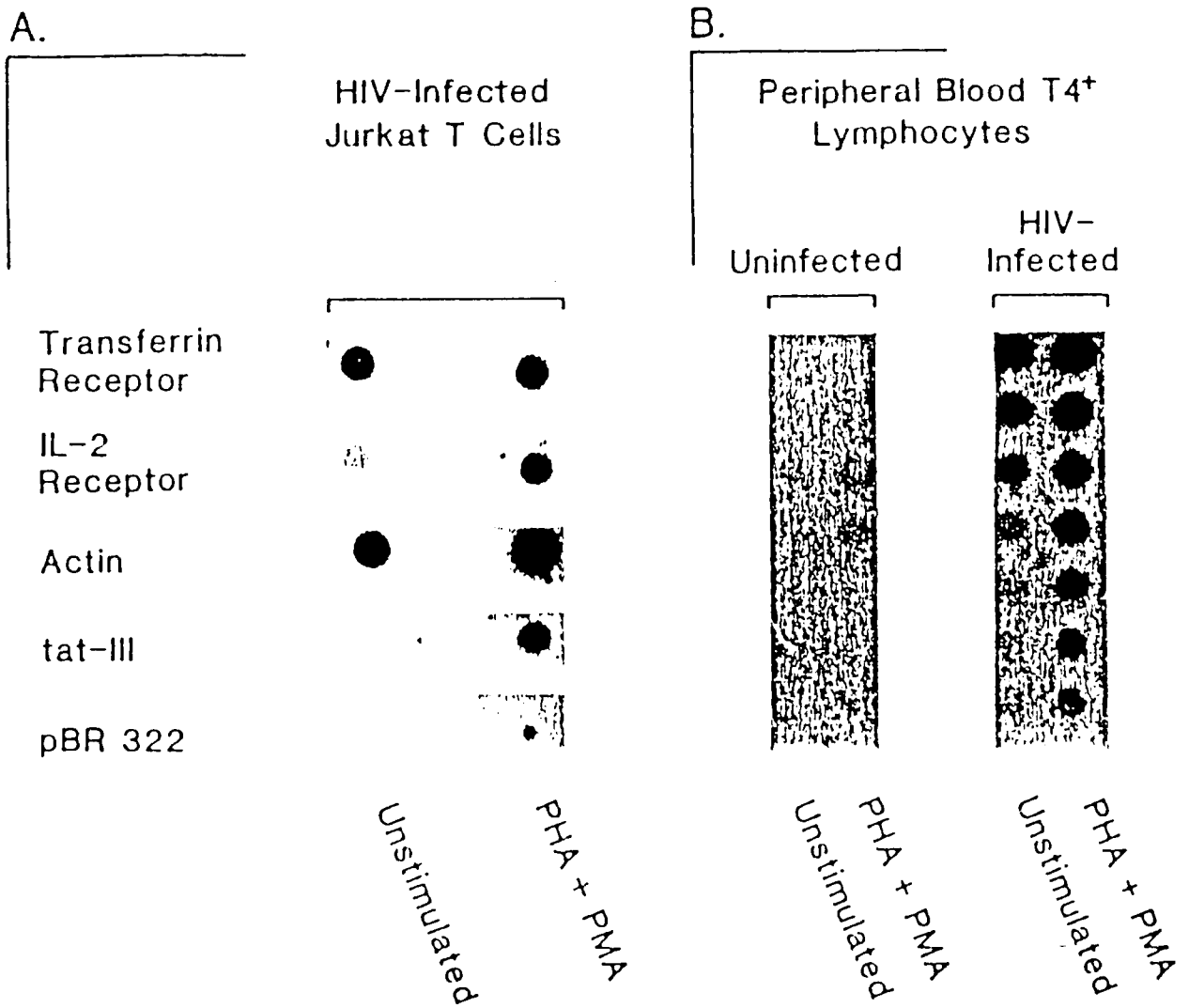



**FIGURE 19: Stimulation of Viral Gene Expression by Mitogens.**
(A) Nuclear transcription analysis of mitogen induced gene expression in HIV-1 infected Jurkat cells. (B) Dot blot analysis of HIV-1 RNA levels in infected $CD4^+$ peripheral blood lymphocytes following mitogen activation.

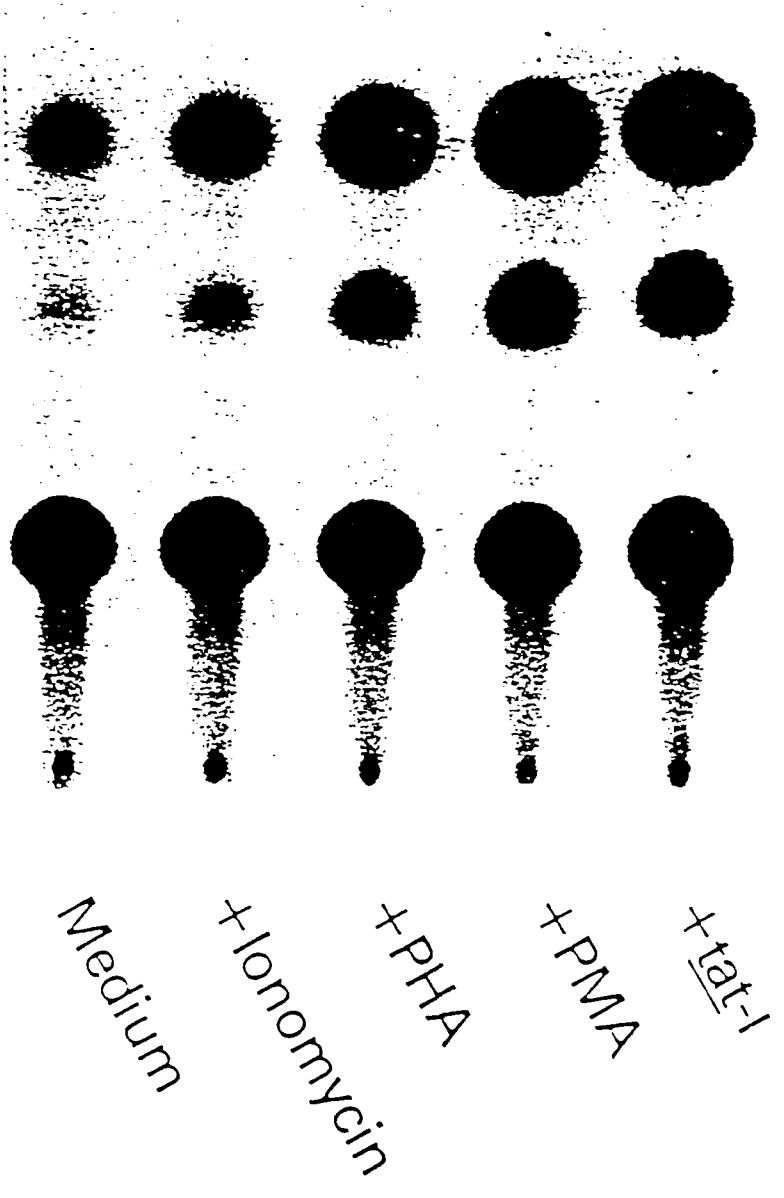



**FIGURE 20: Mitogen Stimulation of the HIV-1 LTR.**
The ability of several mitogens to stimulate CAT production was measured in Jurkat cells transfected with plasmid constructs containing a series of deletion mutations in LTR linked to the CAT gene.

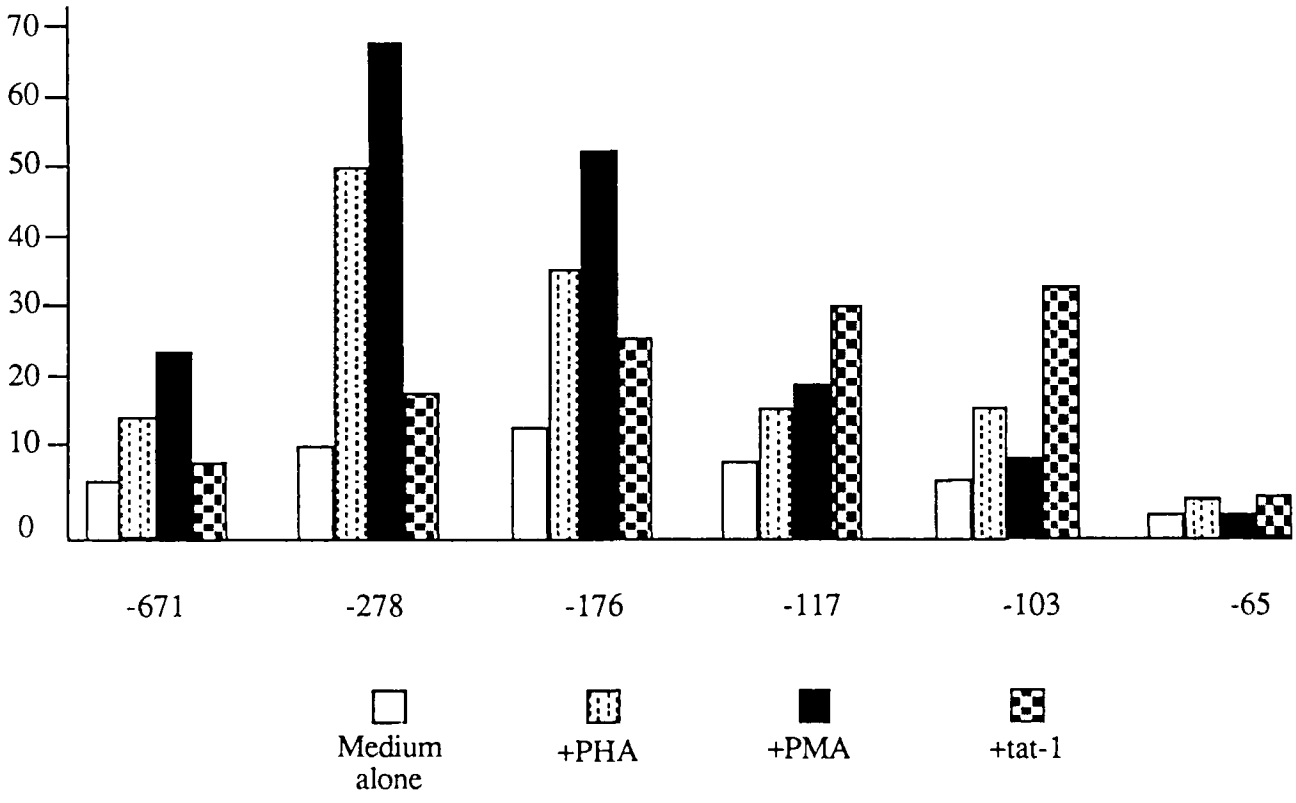



**FIGURE 21: CAT Activity of the HIV-1 LTR Deletion Mutants in the Presence or Absence of PHA, PMA, or tat-I.**

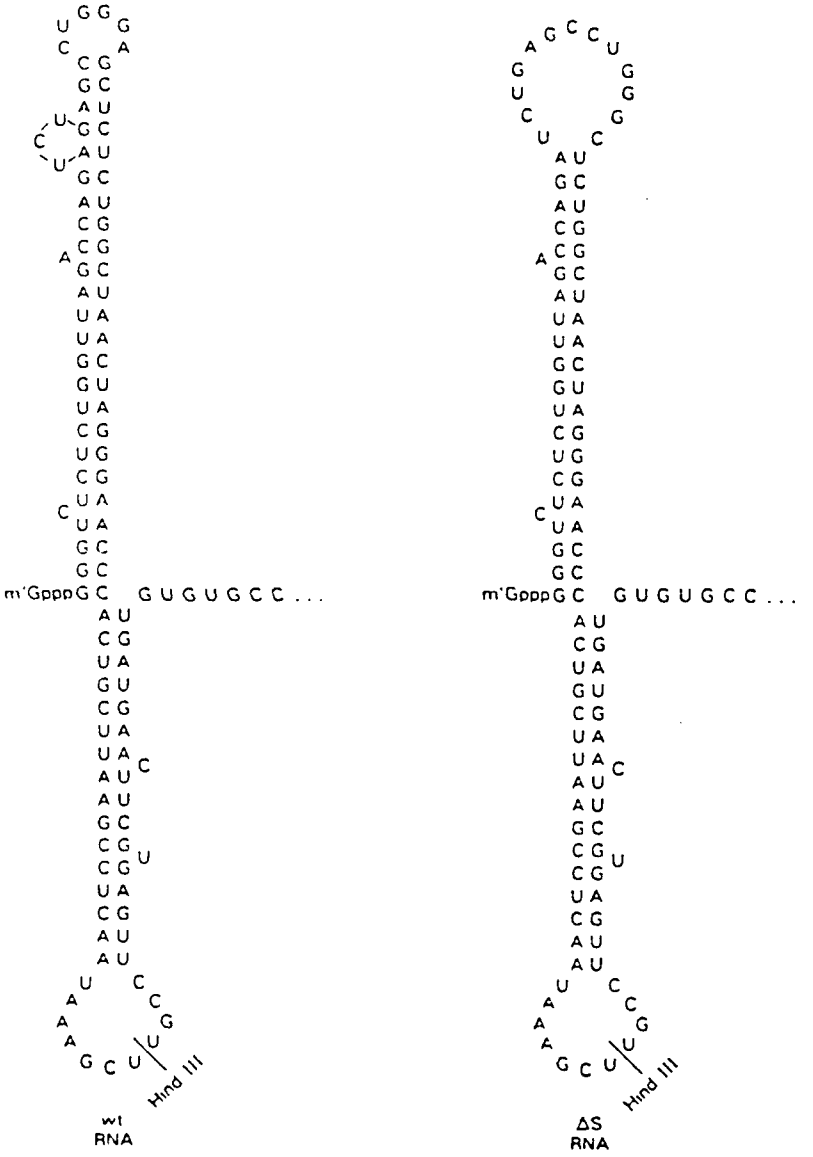



FIGURE 22: Predicted Structures of the Wild Type and Mutant HIV-1 (ΔS) TAR mRNAs.

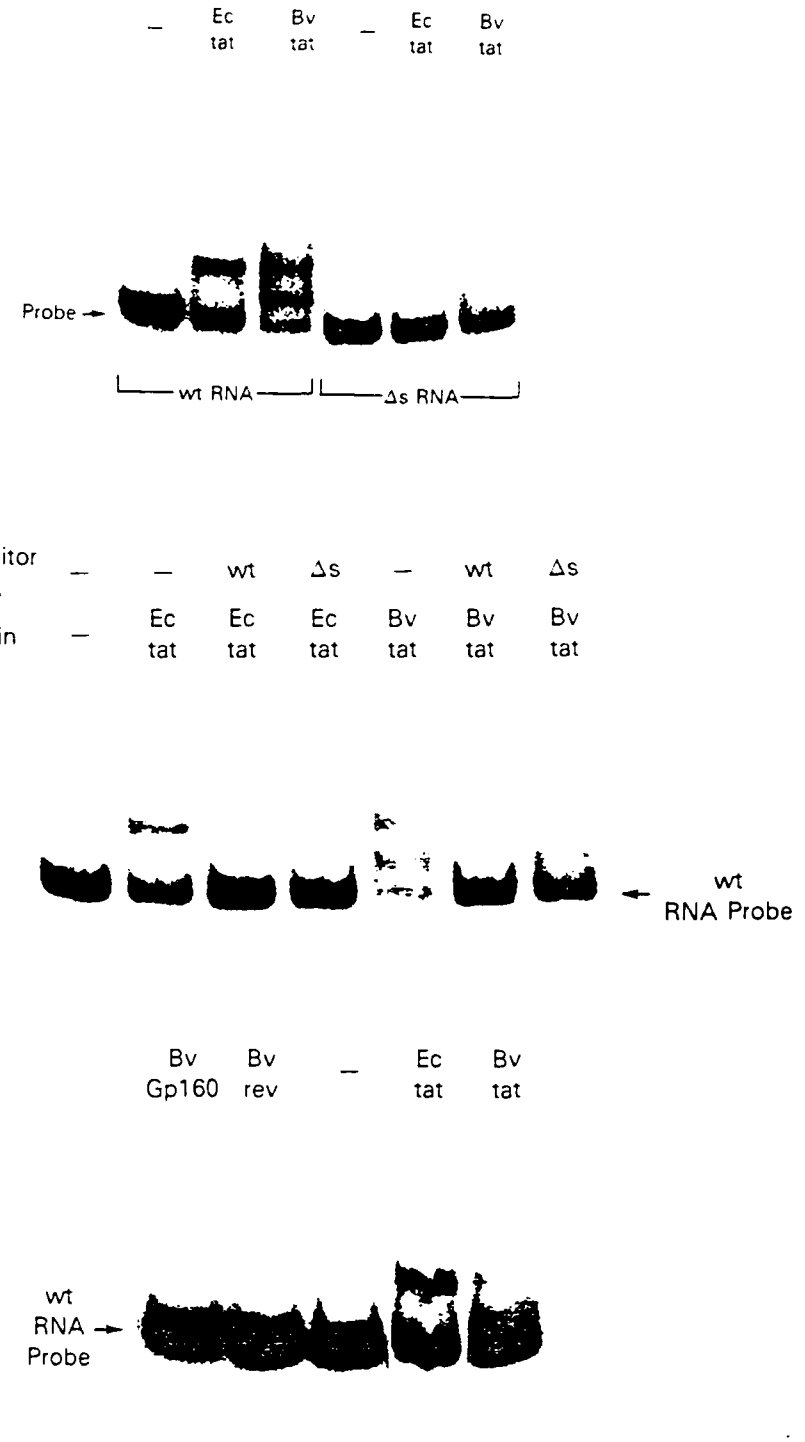



**FIGURE 23: Direct Binding of tat to HIV-1 TAR Transcripts.**

RNA transcripts of T7-TAR-CAT or the ΔS mutant were labeled with $^{32}P$ and incubated in the presence or absence of recombinant tat protein from E. coli or baculovirus expression systems. The resulting complexes were analyzed by gel shift assays of RNA mobility on polyacrylamide gels.

**(A)** Gel Shift Assay of $^{32}P$ RNA transcripts of wild type or mutant (ΔS) in the presence or absence of recombinant tat protein.

**(B)** Gel Shift Assay showing competition of unlabeled RNA transcripts with $^{32}P$ RNA transcripts for binding to recombinant tat protein.

**(C)** Gel Shift Assay of $^{32}P$ transcripts of wild type TAR with recombinant gp160, rev, or tat.

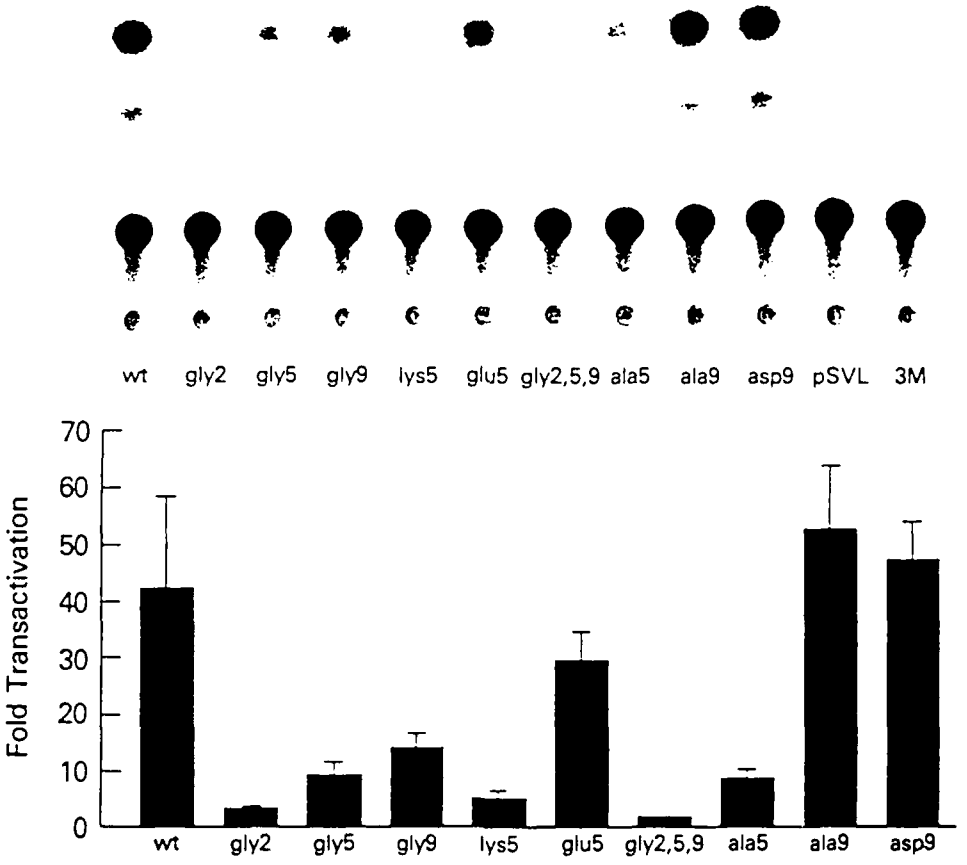



**FIGURE 24: Functional Analysis of Missense Mutations.**
Tat-expressing cDNA clones (1 μg), under the control of the SV40 late promoter, were cotransfected with the plasmid C15-CAT (10 μg) containing the HIV-1 LTR linked to the CAT gene as described in Materials and Methods. A histogram illustrates the average values obtained from three independent sets of transfections. The fold transactivation for wild-type and mutant clones was calculated from the percentage conversion relative to the value obtained with C15-CAT cotransfected with the vector $pSV_L$. CAT activities of each sample in the representative CAT assay shown (upper panel) relative to the wild-type (10% conversion) are: wt (1.00); gly2 (0.06); gly5 (0.23); gly9 (0.29); lys5 (0.11); glu5 (0.53); gly2,5,9 (0.02); ala5 (0.18); ala9 (0.88); asp9 (1.02); $pSV_L$ (0.02); and 3M (0.01), indicating the promoterless construct, pCAT3M.

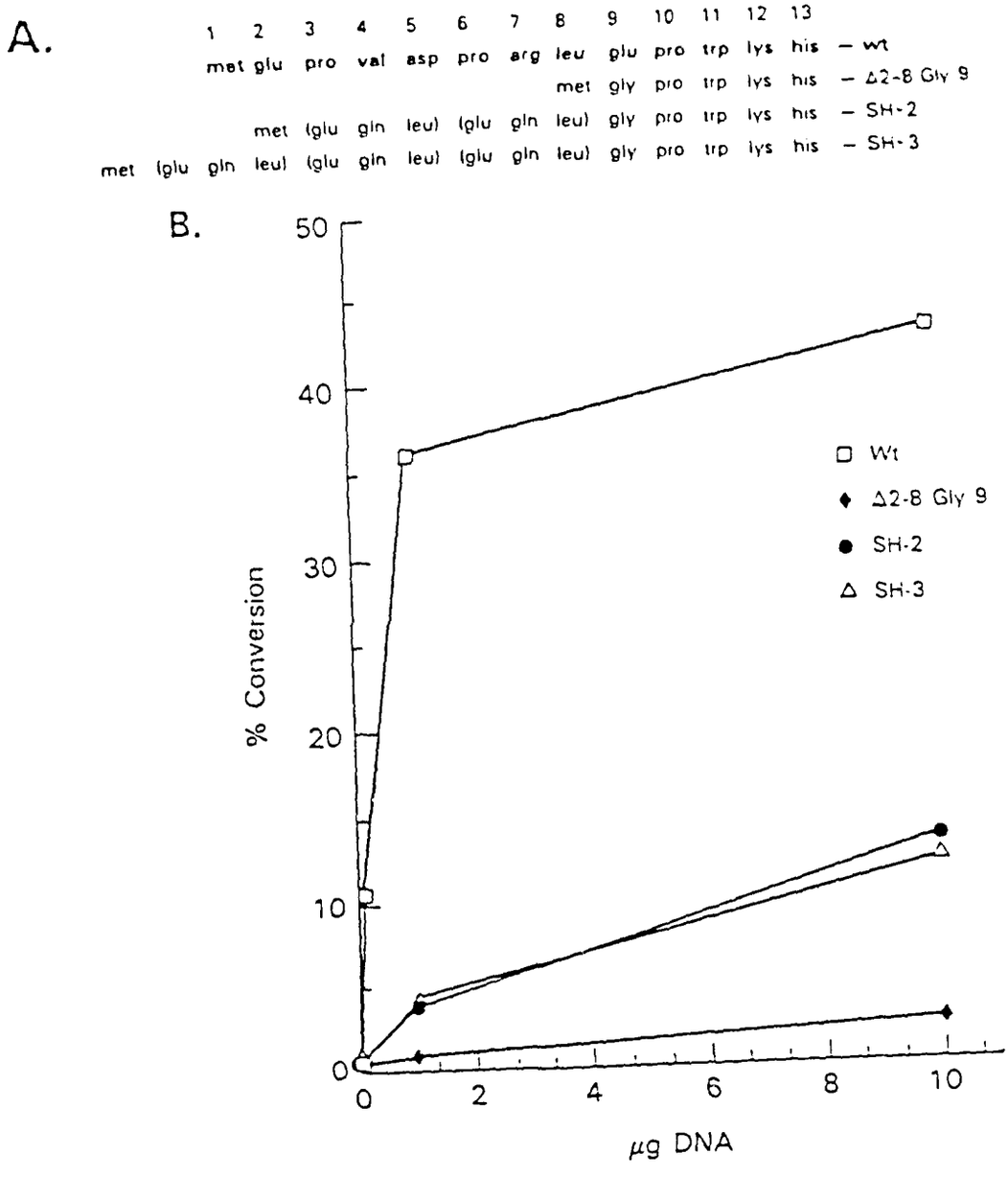



**FIGURE 25: Truncation and Replacement of the Amino Terminal Domain.**
(A) Mutants generated by using the oligonucleotide cassettes as described in Materials and Methods. SH-2 encodes two repeats of the sequence Glu-Gln-Leu, which could form two turns of an acidic-amphipathic helix. SH-3 contains three repeats. The truncation mutant, Δ2-8Gly9 deletes amino acid residues 2-8. All three mutants retain the Gly at position 9 from the original gly2,5,9 construct. (B) CAT activity of wild-type and mutant constructs is shown as a function of input plasmid DNA.

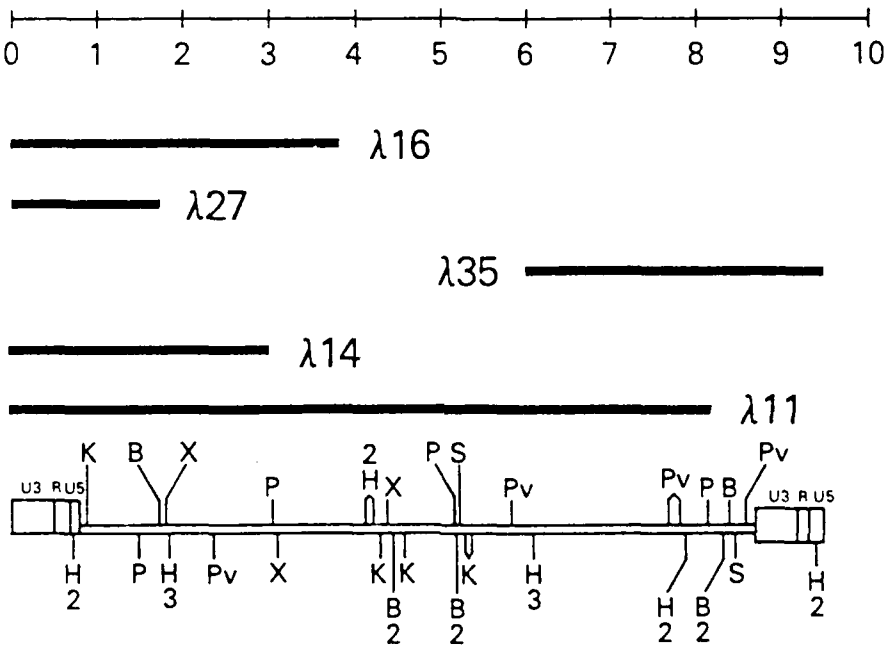



**FIGURE 26: Restriction Enzyme Map of the $SIV_{MAC}$ Genome.**
Five lambda clones were isolated from a genomic library obtained from the $SIV_{MAC}$ infected cell line K6 W. The solid bars represent the individual clones. The endonuclease map depicted in the lower part of the figure was derived by computer analysis of the DNA sequence obtained from four phage clones (16, 27, 35, and 11). The sequence is shown in Figure 32.

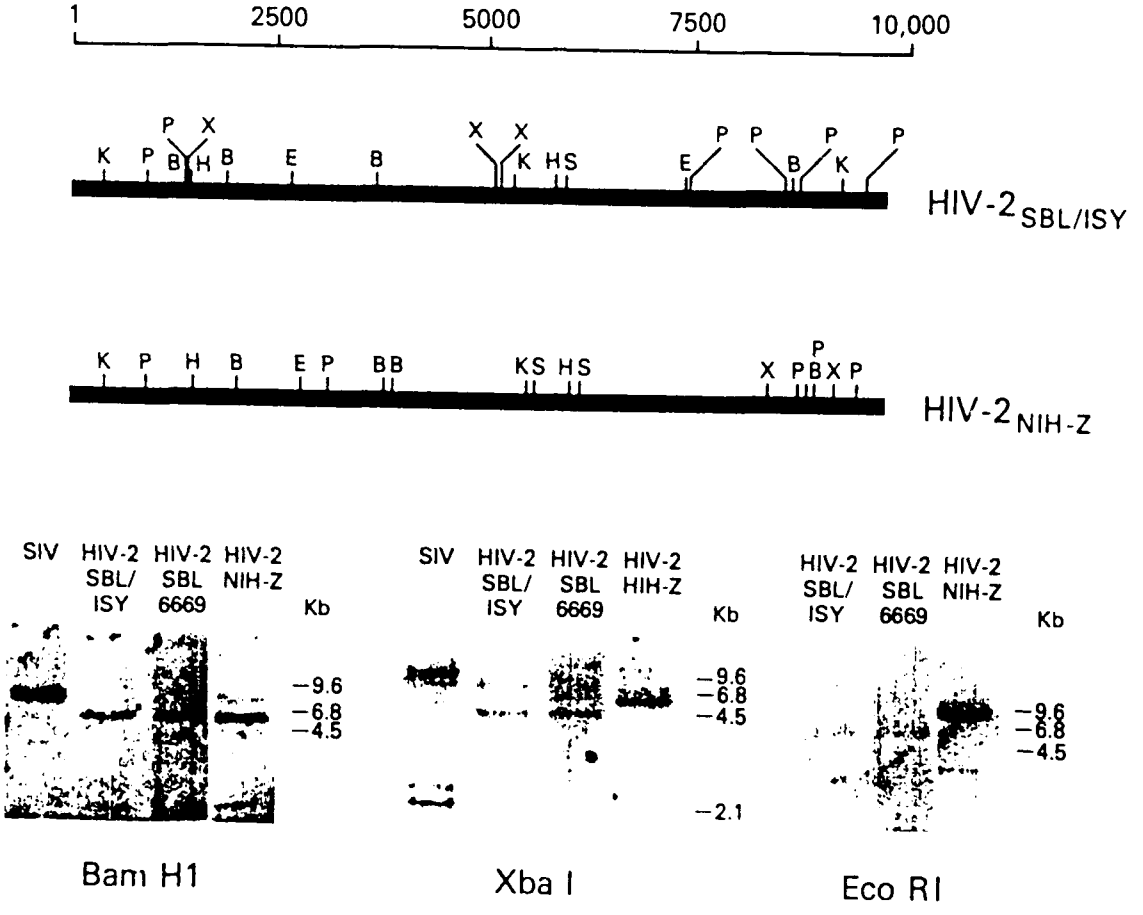



**FIGURE 27: Restriction Enzyme Maps of the HIV-2 Genome.**
The upper portion of the figure represents the endonuclease restriction maps of the proviral HIV-2 clones obtained from the viral isolates HIV-$2_{NIH-Z}$ and HIV-$2_{SBL6669(ISY)}$. The lower portion represents the results of Bam HI, Xba I, and Eco RI cleavage of the genomic DNAs of the HIV-$2_{NIH-Z}$, HIV-$2_{SBL6669}$, HIV-$2_{ISY}$, and $SIV_{MAC251}$ infected cell lines. K=Kpn I, P=Pst I, B=Bam HI, X=Xba I, H=Hind III, E=Eco RI, S=Sac I.

# GENOMIC STRUCTURE OF HUMAN AND NON HUMAN PRIMATE RETROVIRUSES

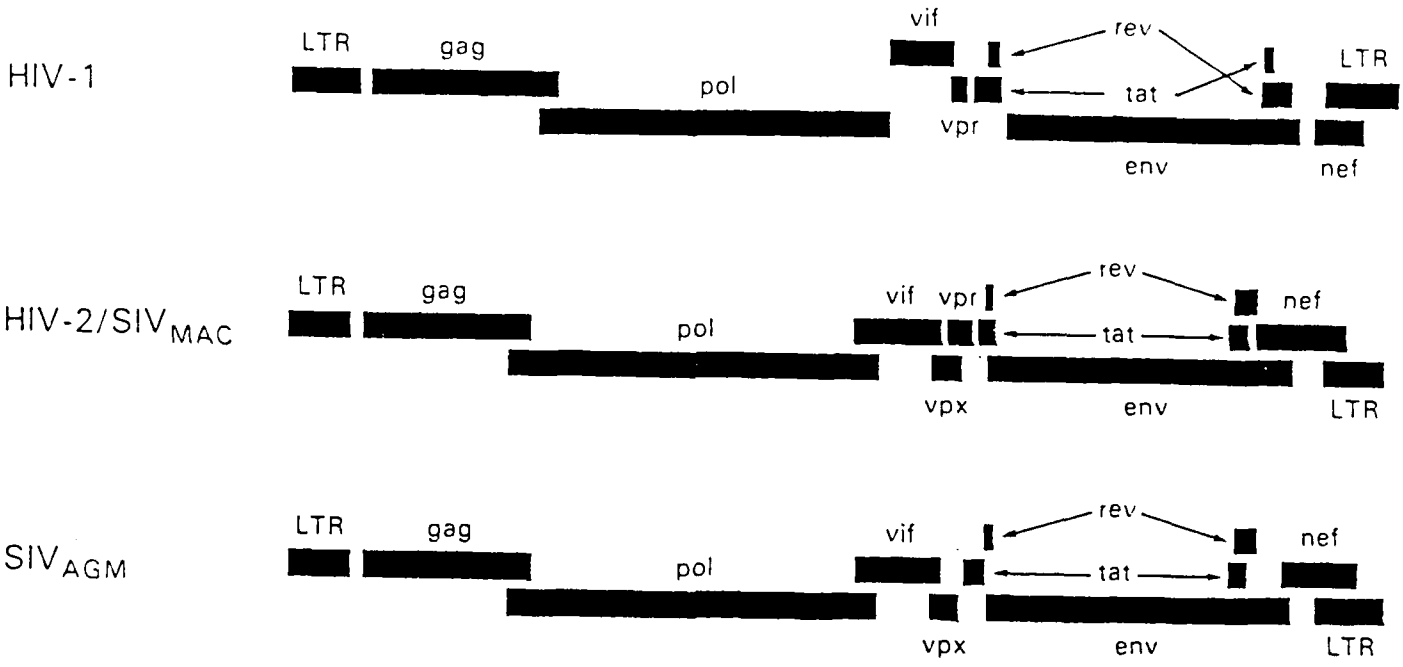
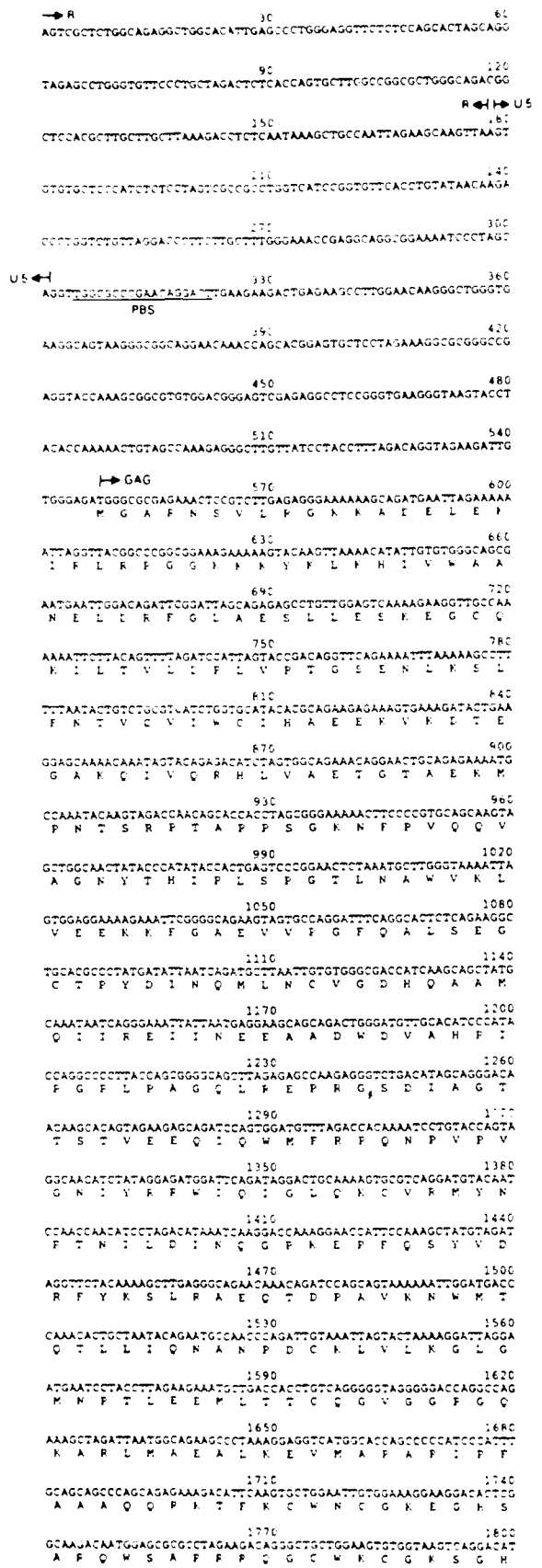



**FIGURE 28: Genomic Organization of HIV-1, HIV-2/SIV$_{MAC}$, and SIV$_{AGM}$.** The bars represent open reading frames corresponding to the various genes, with the exception of the LTRs.

| | | | |
|---|---|---|---|
| gag | = core proteins | vif | = virus infectivity gene |
| pol | = polymerase gene | vpr | = unknown function |
| vpx | = p16, unknown function | tat | = p 14, transactivation gene |
| rev | = p19, regulatory gene | env | = envelope protein |
| nef | = p27, retrovirus inhibitory factor | LTR | = long terminal repeat unit |



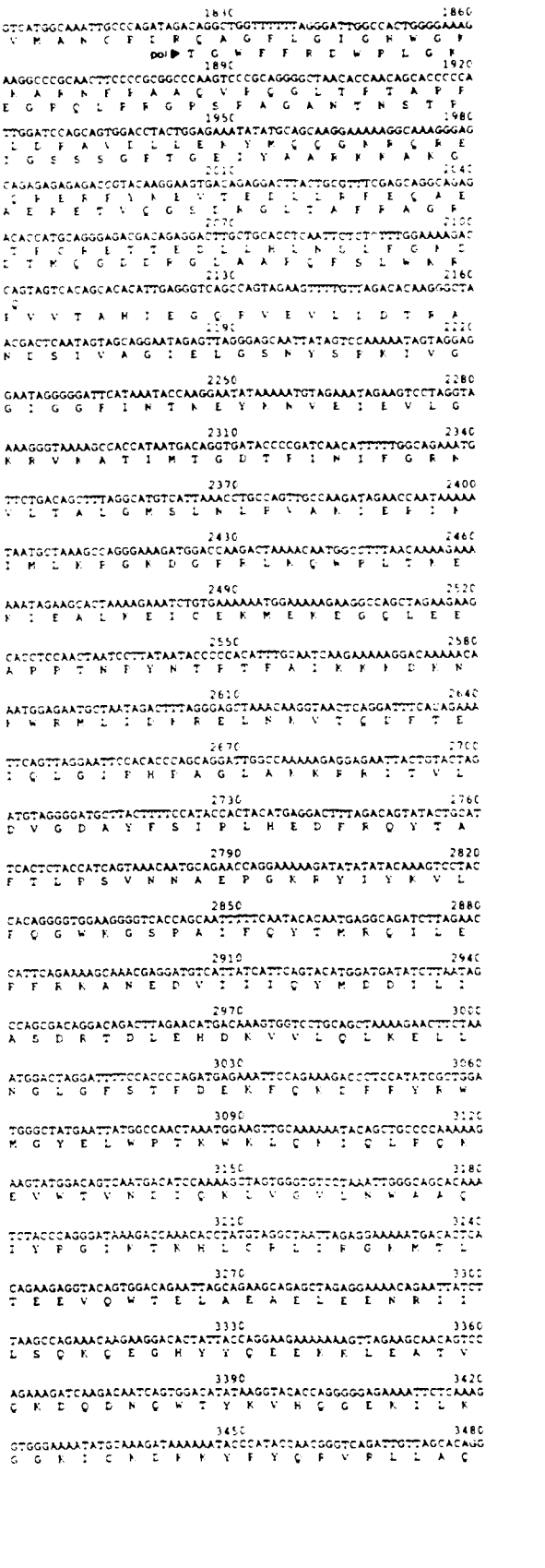




FIGURE 29: The Complete Nucleotide Sequence of the HIV-2$_{NIH-Z}$ Genome.

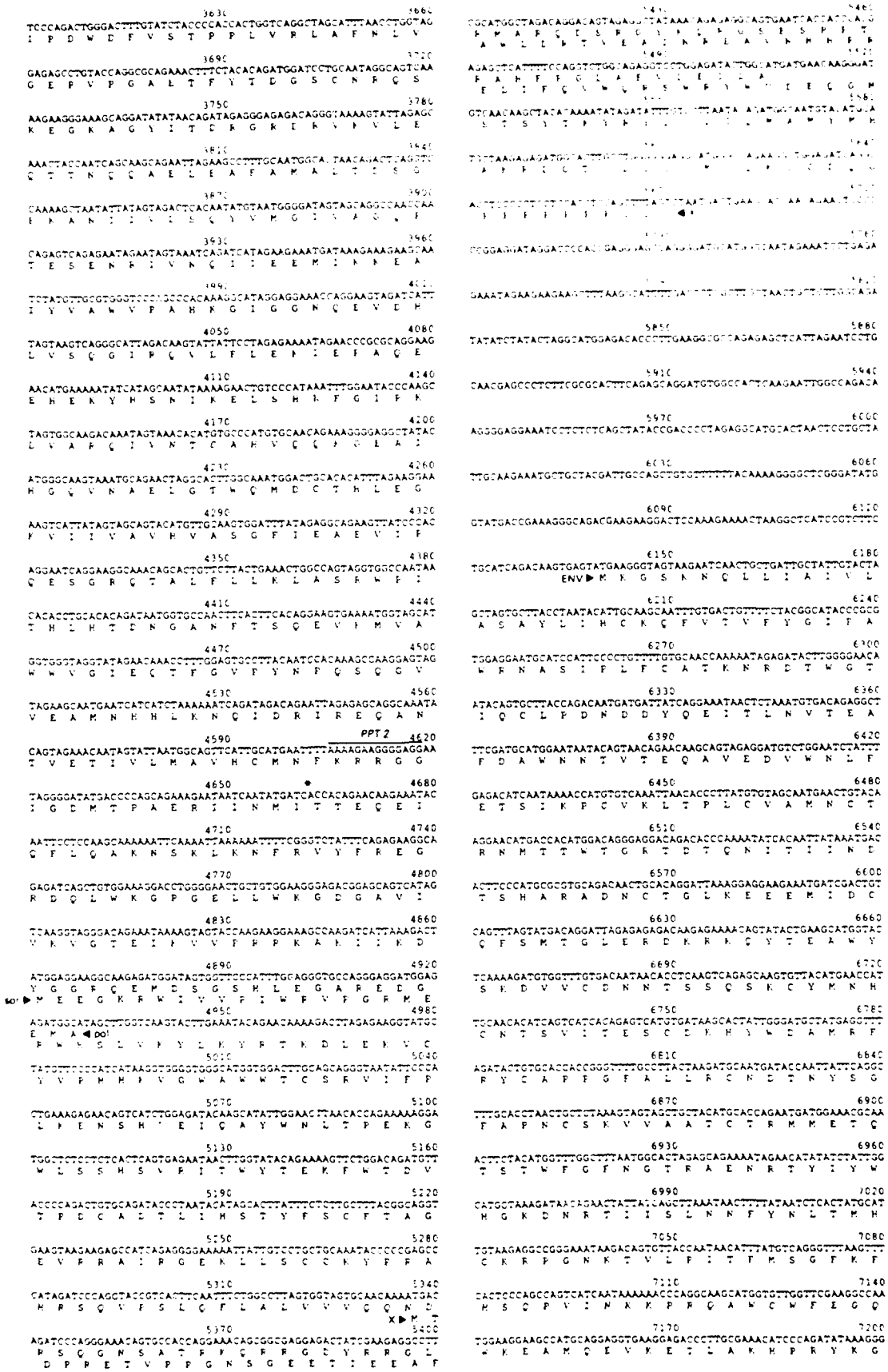


FIGURE 29: The Complete Nucleotide Sequence of the HIV-2$_{NIH-Z}$ Genome.

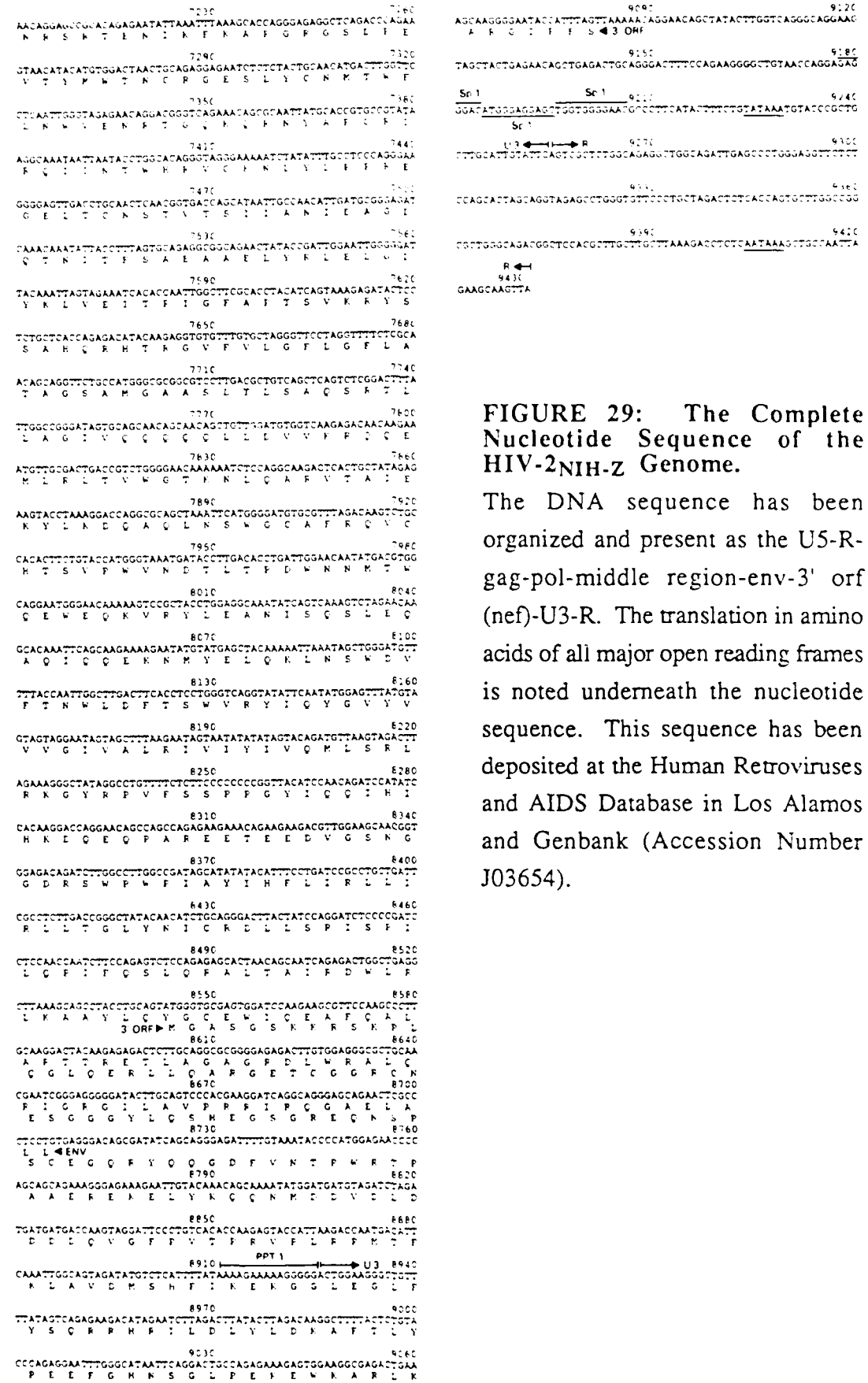


**FIGURE 29: The Complete Nucleotide Sequence of the HIV-2$_{NIH-Z}$ Genome.**

The DNA sequence has been organized and present as the U5-R-gag-pol-middle region-env-3' orf (nef)-U3-R. The translation in amino acids of all major open reading frames is noted underneath the nucleotide sequence. This sequence has been deposited at the Human Retroviruses and AIDS Database in Los Alamos and Genbank (Accession Number J03654).

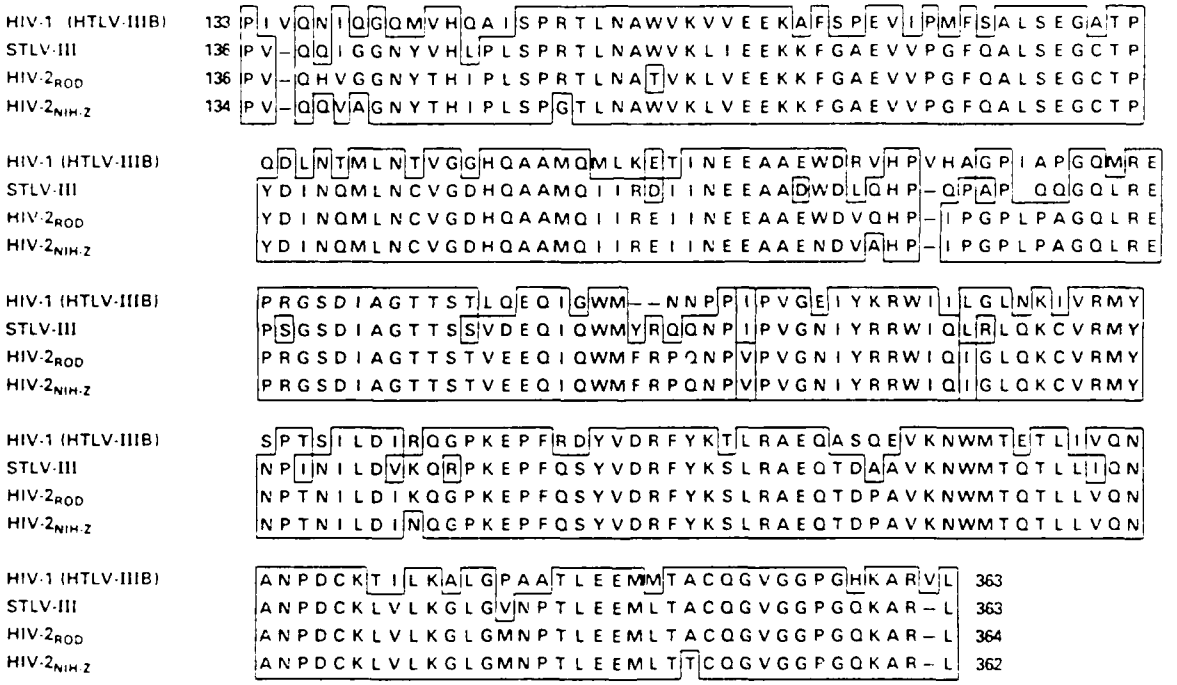



FIGURE 30: Comparison of the Major Core Proteins of HIV-1, HIV-2, and $SIV_{MAC}$.

The amino acids of the major core proteins (p24 - p28) of the HIV-1, SIV, and HIV-2 retroviruses were aligned. The boxed regions indicate amino acids that are identical among all four viral gag proteins.

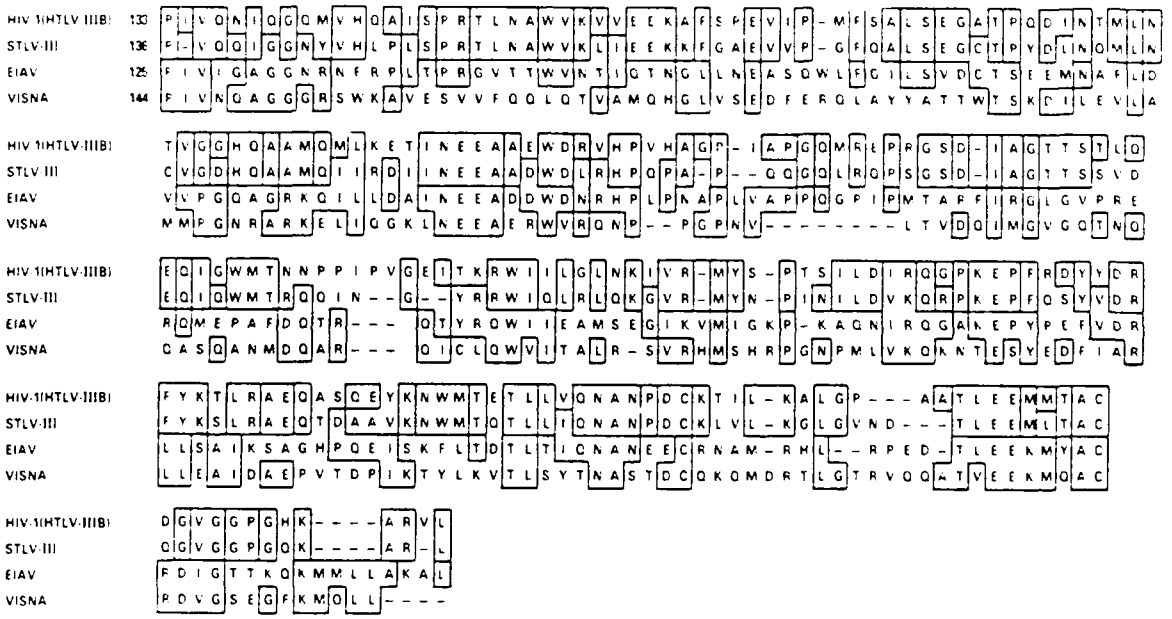



FIGURE 31: Comparison of the Major Core Proteins of HIV-1, SIV, EIAV, and VISNA Retroviruses.

The amino acids of the major core proteins of HIV-1, $SIV_{MAC}$, EIAV, and VISNA retroviruses were aligned. The boxed regions indicate amino acids that are identical among all four viral gag proteins.

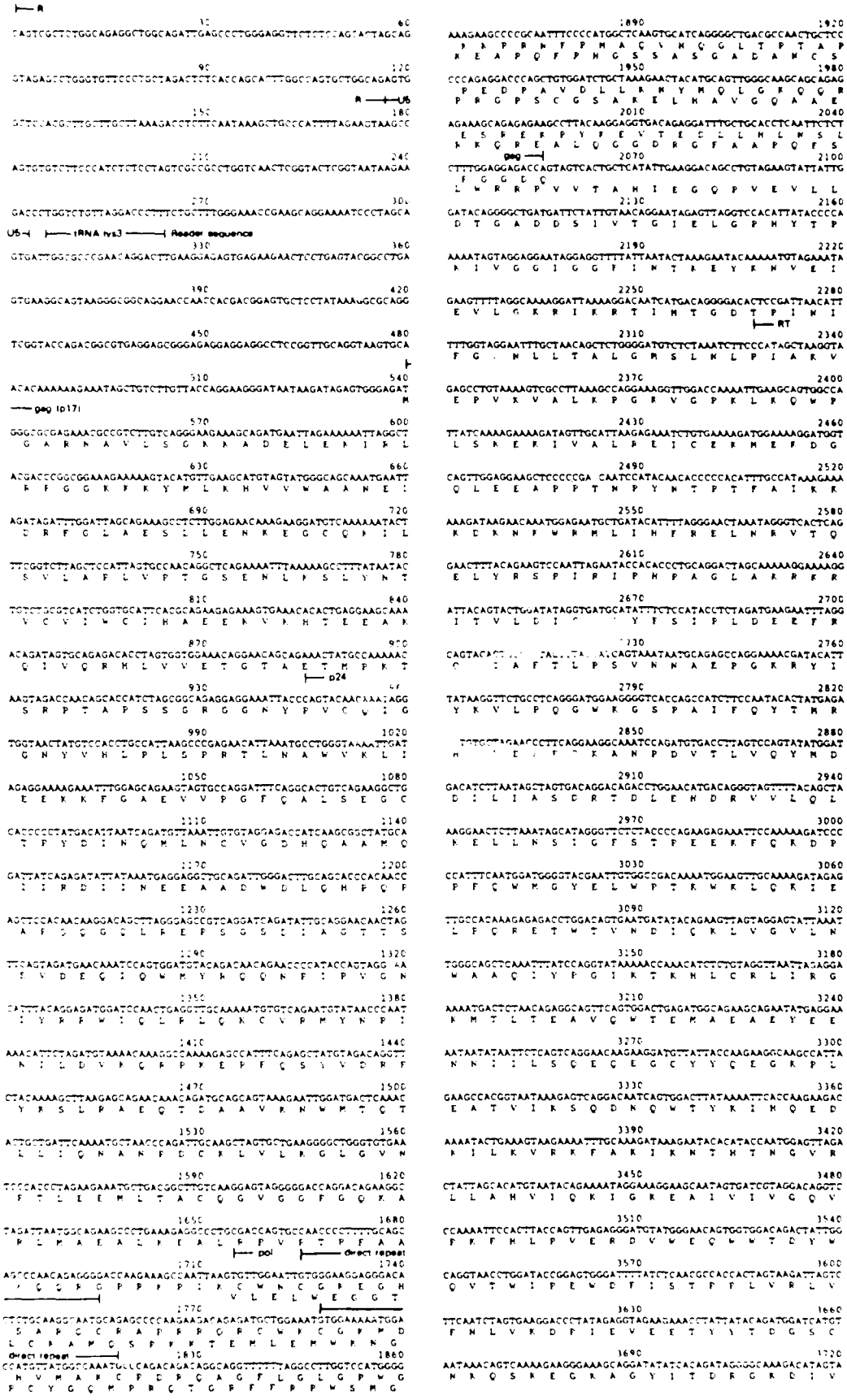



Figure 32: Nucleotide Sequence of $SIV_{MAC}$

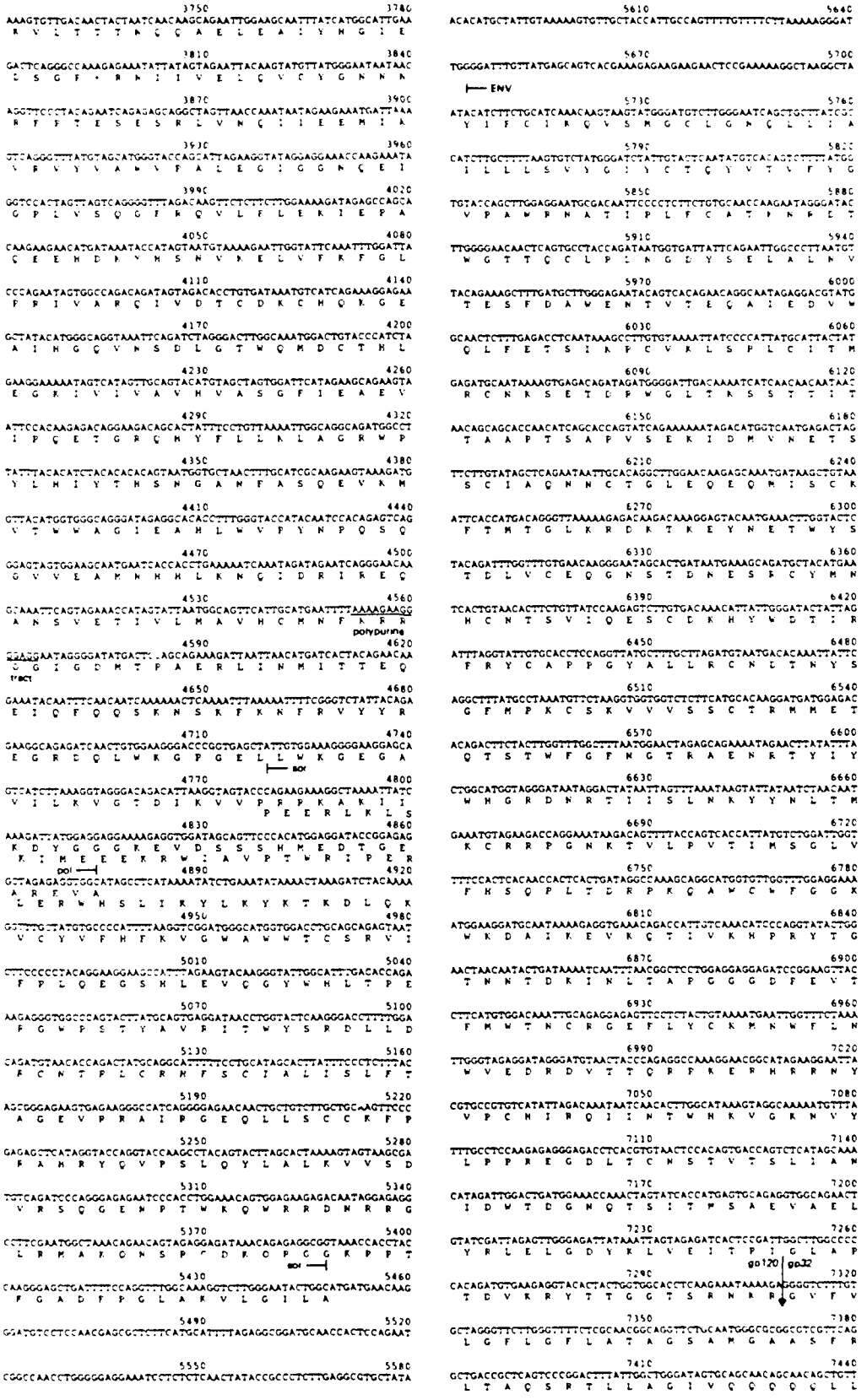



Figure 32: Nucleotide Sequence of $SIV_{MAC}$

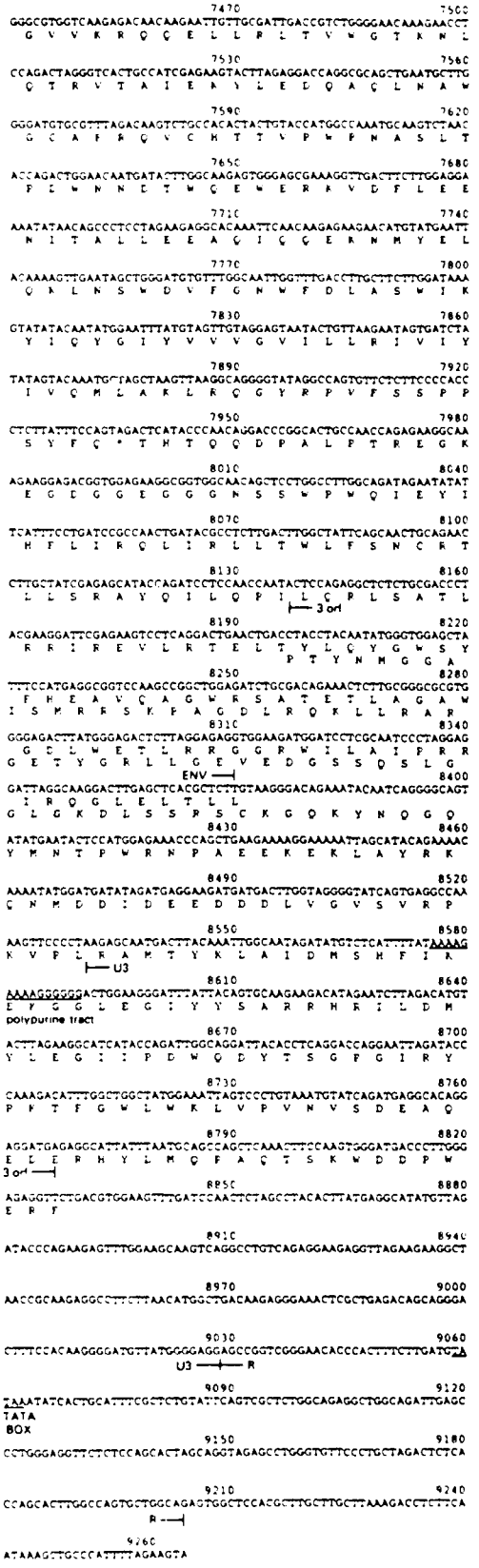



**FIGURE 32: The Complete Nucleotide Sequence of $SIV_{MAC}$.**
The DNA sequence of the complete provirus genome of $SIV_{MAC}$ was determined using the dideoxy chain termination method of Sanger on single and double stranded DNA. The sequence has been numbered from the beginning of the R region at the 5' end of the provirus to the end of the R region at the 3' end of the proviral DNA. The major gene regions are indicated throughout the length of the sequence. The translation in amino acids of all major open reading frames is presented underneath the nucleotide sequence.

```
BASE COUNT     3291 a   1940 c   2396 g   2009 t
ORIGIN
        1 Agtcgctctg cggagaggct ggcagattga gccctgggag gttctctcca gcactagcag
      mRNA ->
     R rpt ->
       61 gtagagcctg ggtgttccct gctggactct caccagtgct tggccggcgc tgggcagacg
      121 gctccacgct tgcttgctta aaagacctct tAATAAAgct gccagttaga agcaagttaa
                                                                  <- R rpt
      181 gtgtgtgttc ccatctctcc tagtcgccgc ctggtcattc ggtgttctcc tgagtaacaa
      241 gaccctggtc tgttaggacc cttcttgctt tgggaaaccg aggcaggaaa atccctagca
                                                                      <-LTR
      301 ggttggcgcc cgaacaggga cttgaggaag actgagaagc cttggaacac ggctgagtga
            -> Lys-tRNA pbs <-
      361 aggcagtaag ggcggcagga acaaaccacg acggagtgct cctagaaagg cgcaggccaa
      421 ggtaccaaag gcggcgtgtg gagcgggagt caagaggcct ccgggtgaag gtaagtacct
      481 acaccaaaaa ctgtagccgg aaaaggcttg ttatcctacc tttagacagg tagaagattg
      541 tgggagATGg gcgcgaaaaa ctccgtcttg agagggaaaa aggcagatga attagaaaaa
  gag cds start ->
      601 attaggttac ggcccggcgg gaagaaaaaa tacagattaa aacatattgt gtgggcagcg
      661 aatgaattgg acagattcgg attaacagag agcctgttgg agtcaaaaga aggttgccaa
      721 aaaattattt cagttttaga accattagta ccaacagggt cagaaaattt aaaaagcctt
      781 tataatacta cctgcgtcat ttggtgcttg cacgcagaag agaaagtgaa agatactgaa
      841 gaagcaaaaa gaatagtagg gagacatcta gtggcagaaa cagaaactgc agagaaaatg
      901 ccaaatataa gtagaccaac agcaccacct agcgggaaag ggggaaactt ccccgtgcaa
      961 caaataggcg gcaactatgt ccatctgccg ctgagtcccc gaaccctaaa tgcttgggta
     1021 aagttagtag aggagaaaaa gttcggggca gaagtagtgc cgggatttca ggcactctca
     1081 gaaggctgca cgccctatga tattaatcaa atgcttaatt gtgtgggcga ccatcaagca
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     1261 gggacaacaa gcacagtaga ggaacagatc gaatggatgt ataggcaaga aaatcctgta
     1321 ccagtaggaa acatctatag gagatggatc cagataggac tgcagaagtg tgtcagaatg
     1381 tacaatccaa ccaacattct agacataaaa caaggaccaa aagagtcgtt ccaaagctat
     1441 gtggatagat tctacaaaag cttaagggca gaacagacag atgcagcagt gaagaattgg
     1501 atgacccaga cgctgctagt gcaatcgaac ccagactgta agttagtact aaagggacta
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     1681 tttgcagcag cccaacagaa aagggcaatt aagtgttgga attgtggaaa ggaagggcac
     1741 tcggcaagac aatgccgagc gcctagaaga cagggctgct ggaaatgtgg caagtcagga
     1801 cacatcatgg caaactgccc agaTAGacag gctggttttt tagggcttgg accatgggga
   pol start (NH2-terminus uncertain)->
     1861 aagaagcccc gcaacttccc cgtggtccca agttcgcagg ggctaacacc aacagcaccc
     1921 ccaatggatc cagcagtgga cctactggag aagtacatgc agcaagggag aaaacagaga
     1981 gagcagagac aaagaccata caaagaagtg acagaggact tgctgcatct cgagcaagga
     2041 gagacaccac acagagagac gacagaggac ttgctgcacc tcaattctct ctttggaaac
     2101 gaccagTAGt cacagcatac attgaggatc agccagtaga agttttacta gacacagggg
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     2161 ctgacgactc aatagtagca ggaatagagt tagggagcaa ttatagtcca aaaatagtag
     2221 ggggaatagg aggattcata aataccaaag aatataaaga tgtagaaata agagtgctaa
     2281 ataaaaaggt aagagccacc ataatgacag gtgatacccc aatcaacatt tttggcagaa
     2341 atatcctgac agccttgggc atgtcattaa atttaccagt cgccaaaata gaaccagtaa

     2401 aagtaacatt aaagccagga aaagatgggc caaaacaaag acaatggccc ttaacaagag
     2461 aaaaaataga agcactaaga gaaatctgtg aaaaaatgga aagagaaggt cagctagaag
     2521 aagcgcctcc aactaatccc tataataccc ctacatttgc aattaagaaa aaggacaaaa
     2581 acaaatggag gatgctgata gattttagag aactaaacaa ggtaactcaa gatttcacag
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     2701 tagatgtagg agatgcctac ttttccatac ccctatatga ggattttaga cagtatactg
     2761 ca:ttactct gccatcagta aacaatgcag aaccaggaaa aagatatata tacaaagtct
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     2881 aaccattcag aaaagcaaac ccagatgtca ttatcgttca gtacatggat gatatcttaa
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     3061 ggatgggcta tgaactgtgg ccaaccaaat ggaaattgca aaaaatacaa ttgccccaaa
     3121 aggaagtatg gacagttaat gacatccaga aactagtggg tgtcctaaac tgggcggcac
     3181 aaatctaccc aggaataaag accaaacact tatgtaagct aattagagga aagatgacac
     3241 ccacggaaga agtacagtgg acagaattag cagaagcaga gctggaggaa aacaaaatta
```

FIGURE 33: The Complete Nucleotide Sequence of HIV-$2_{ISY}$.

```
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 3361 ttcaaaagga tcaagacaat cagtggacat ataaagtaca ccagggagaa aaaattctaa
 3421 aagtaggaaa atatgcaaag ataaaaaata cccataccaa cggggtcagg ttgttggcac
 3481 aggtagttca gaaaatagga aaagaagcac tagtcatttg gggacgaata ccaaaatttc
 3541 acctaccagt agaaagagag acctgggaac agtggtggga taactattgg caagtgacat
 3601 ggatcccaga ctgggacttc gtatccaccc caccgttggt caggttagca tttaacctgg
 3661 taaaagatcc tataccaggc gcagagacct tctacacgga tggatcttgc aataggcaat
 3721 caaaagaggg aaaagcagga tatataacag atagaggaaa agacaaagta aggatattag
 3781 agcaaactac caaccaacaa gcagaattag aagcctttgc aatggcagta acagactcag
 3841 gtccaaaagt caatattgta gtagactcac agtatgtaat gggaatagta acaggccaac
 3901 cggctgaatc agagagtaga atagtaaata aaattataga agagatgata aaaaaggaag
 3961 caatctatgt tgcatgggtc ccggcccaca aaggcatagg aggaaatcaa gaaattgacc
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vif start->
 4921 gagaaatggc aTAGccttat caagtatcta aaatacagaa caaaagatct agaacaggtg
                    <- pol cds end
 4981 cgctatgttc cccaccataa ggtggggtgg gcatggtgga cttgcagcag ggtaatattc
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 5281 gcccataagt ccaaggtacc gtcactccaa tttctggcct tagtagtagt acagcaaaAT
                                                    vpX cds start ->
 5341 Gacaaacccc agagagacaa taccaccagg aaacagtggc gaagaaacta tcgaagaggc
 5401 cttcgattgg ctagacagga cggtagaagc cataaacaga gaggcagtga accacctgcc
 5461 cagggagctt attttccagg tgtggcaaag gtcctggaga tactggcaTG Atgagcaagg
                                                          <- vif cds
 5521 gatgtcacga agctacacaa agtatagata tttgtgctta atgcagaaag ctgtgttcat
 5581 gcatttcaag aaagggtgca cttgccgggg ggaaggacat gggccaggag ggtggagatc
 5641 aggacctccc cctcctcctc ccccaggttt agtcTAATGa ctgaagcacc agcagagttt
                                          <- vpX cds end
                                 vpR cds start ->
 5701 cccccggagg atgggacccc accgagggaa ccaggggatg agtgggtaat agaaattctg
 5761 agagaaataa aggaagaagc tttaaagcat tttgaccctc gcttgctaac tgctcttggc
 5821 tactatatct atactagacA TGgagacacc cttgaaggcg ccagagagct cattagggtc
           tat cds start ->
 5881 ctacaacgag ccctcttcac gcacttcaga gcaggatgtg gccactcaag aattggccaa
 5941 ccaaggggaa gaaatcctct ctcagctata ccgaccccta gaaacatgca aTAAcaaatg
                                                             <- vpR
 6001 cttttgtaag gggtgctgct tccattgcca gctgtgtttt ttaaacaagg ggctcgggat
 6061 atgttATGac cgaaagggca gacgaagaag gagtccgaag aaaactaagg ctcattcgtc
rev cds start ->
 6121 tcctgcatca gacaaGTgag tacaATGagt ggtaaaattc agctgcttgt tgcctttctg
(tat, rev, nef) 5' sj /\
                   env cds start ->
 6181 ctaactagtg cttgcttaat atattgcacc aaatatgtga ctgttttcta tggagtaccc
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 6361 gcttttgacg catgggataa tatagtaaca gaacaagcag tagaagatgt ctggaatcta
 6421 tttgagacat caataaaacc atgtgtcaaa ttaacgcctt tatgtgtaac aatgaactgt
 6481 aacgcaagta cagagagcgc agttgcaact acaagcccat ctggacctga tatgataaat
```

FIGURE 33: The Complete Nucleotide Sequence of HIV-$2_{ISY}$.

```
6541 gatactgatc catgcataca attgaacaat tgctcaggac tgagggagga agacatggtc
6601 gagtgtcagt tcaatatgac aggactagag ttagataaga aaaaacagta tagtgaaacc
6661 tggtactcaa aagatgtggt ttgtgaatca gataacagca cagaccgaaa aagatgttac
6721 atgaaccatt gcaacacatc agtcatcaca gagtcatgtg acaagcacta ttgggatgct
6781 atgagattta gatactgtgc accaccgggt tttgtcttgc taaggtgcaa tgataccaat
6841 tactcaggct ttgagcccaa ttgctctaaa gtagtagctt ctacatgtac aagaatgatg
6901 gaaacgcaac cttctacttg gcttggcttt aatggcacta gggcagaaaa tagaacatat
6961 atctattggc atggtaggga taacagaact attatcagct taaacaaata ttataatctc
7021 accatacttt gtaggagacc agaaaataaa acagttgtac caataacact catgtcaggc
7081 cgcagatttc actcccagaa gatcatcaat aaaaaaccca ggcaagcatg gtgccggttc
7141 aaaggcgagt ggagggaagc catgcaggag gtgaaacaaa cccttgtaaa acatcccagg
7201 tataaaggaa ccaatgacac aaataaaatt aactttacag caccagaaaa agactcagac
7261 ccagaagtag catatatgtg gactaactgc agaggagaat tcctctattg caacatgact
7321 tggttcctta attgggtaga aaacaagacg ggtcaacagc ataactatgt gccgtgccat
7381 atagagcaaa taattaatac ctggcataag gtagggaaaa atgtatattt gcctcctagg
7441 gaaggagagt tgtcctgcga atcaacagtg accagtatca ttgctaacat tgatgttgat
7501 ggagataacc ggacaaatat tacctttagt gcagaggtgg cagaactata ccgattggaa
7561 ttgggggatt ataaattagt agaagtaaca ccaattggct tcgcccctac agcagaaaaa
7621 agatactcct ctgctccagg gagacataag agaggtgtgc ttgtgctagg gttcctaggt
7681 tttctcacga cagcaggtgc tgcaatgggg gcggcgtctc tgacgctgtc ggctcagtct
7741 cggactttat tccgtgggat agtgcagcaa cagcaacagc tgttggacgt ggtcaagaga
7801 caacaagaaa tgttgcgact gaccgtctgg ggaactaaaa acctccaggc aagagtcact
7861 gctattgaga agtacctagc agaccaggcg cgactaaatt catggggatg tgcgtttaga
7921 caagtctgcc acactactgt accatgggta aatgacacct taacacctga gtggaacaac
7981 atgacatggc aagaatggga acacaaaatc cgcttcctag aggcaaatat cagtgagagt
8041 ttagaacagg cacaaatcca gcaagaaaag aatatgtatg agctgcaaaa gctaaatagc
8101 tgggatgttt ttggcaattg gtttgactta acctcctgga tcaagtatat tcaatatgga
8161 gtcatgatag tagtaggaat agtagctctc agaatagtaa tatatgtagt acaaatgcta
8221 agtagactta gaaagggcta taggcctgtt ttctcttccc cccccggtta tattcaacAG
                                                              3' sj /\
8281 atccatatcc acaaggactg ggaacagcca gacagagaag aaacagaaga agacgttggg
8341 aacgacgttg gaagcagatc ctggccttgg ccgaTAGaat atatacattt cctgatccgc
                                          <- tat cds end
8401 ctgctgatcc gcctcttgac cagactatac aacagctgca gggacttact atccagactc
8461 tacctgatcc tccaaccact cagagactgg ctgagactca aggcagccta cctgcagtAT
                                                   nef cds start ->
8521 Gggtgcgagt ggatccaaga agcgttccag gccctcgcga gggttacaag agagactctt
8581 acgagcgcgg ggaggagctt gtgggggct ctgggacgaa tcggaagggg gatactcgca
8641 gttccacgaa ggatcaggca gggagcagaa attgccctcc tgTGAgggac agagatatca
                                                 <- env cds end
8701 gcaaggagac tttaTGAata ccccatggag aaccccagca acagaaaagg aaaaagaatc
                     <- rev cds end
8761 gtacaggcaa caaaatatgg atgatgtaga ttcagatgat gatgacctag taggggtctc
8821 tgacacatca agagtaccat tgagagcaat gacatataga atggcagtag acatgtcaga
8881 tttaataaaa gataaggggg gactggaagg gatgtattac agtgagagaa gacatagaat
                        3' LTR ->
8941 cctagacata tacttagaaa aggaagaagg gataattcca gattggcaga actatactca
9001 tgggctagga gtaaggtacc caatgttctt tgggtggcta tggaagctag taccagtaac
9061 tgtcccacaa gaaggggagg acactgagac tctctgctta atgcactcag cacaagtaag
9121 cagatttgat gacccgcatg gggagacact agtctggaag tttgacccca tgctggctca
9181 tgagtacacg acctttattc tatacccaga ggaatttggg cacaagtcag gaatggaaga
9241 agatgactgg aaggcaaaac tgaaagcaag agggatacca tttagtTAAa aacaggaaca
                                                   <- nef cds end
9301 accatacttg gtcaggacag gaagtagcta ctgaaaacag ctgagactgc agggactttc
9361 cagaaggggc tgtaaccagg ggagggacat gggaggagct ggtggggaac gccctcatac
9421 tttctgTATA AAtgtacccg ctgctcgcat tgtattcagt cgctctgcgg agaggctggc
    signal ->                          R rpt ->
9481 agattgagcc ctgggaggtt ctctccagca ctagcaggta gagcctgggt gttccctgct
9541 ggactctcac cagtgcttgg ccggcgctgg gcagacggct ccacgcttgc ttgcttaaaa
9601 gacctcttAA TAAAgctgcc agttagaagc aagtta
      signal ->                            <- mRNA
                                           <- R rpt
```

**FIGURE 33: The Complete Nucleotide Sequence of HIV-$2_{ISY}$.**
The DNA sequence of the provirus was determined using the dideoxy chain termination method of Sanger on double stranded DNA and the chemical modification/cleavage method of Maxam and Gilbert. The major coding regions are indicated. This sequence was copied from the Human Retroviruses and AIDS database, 1990. (Accession Number J04498).

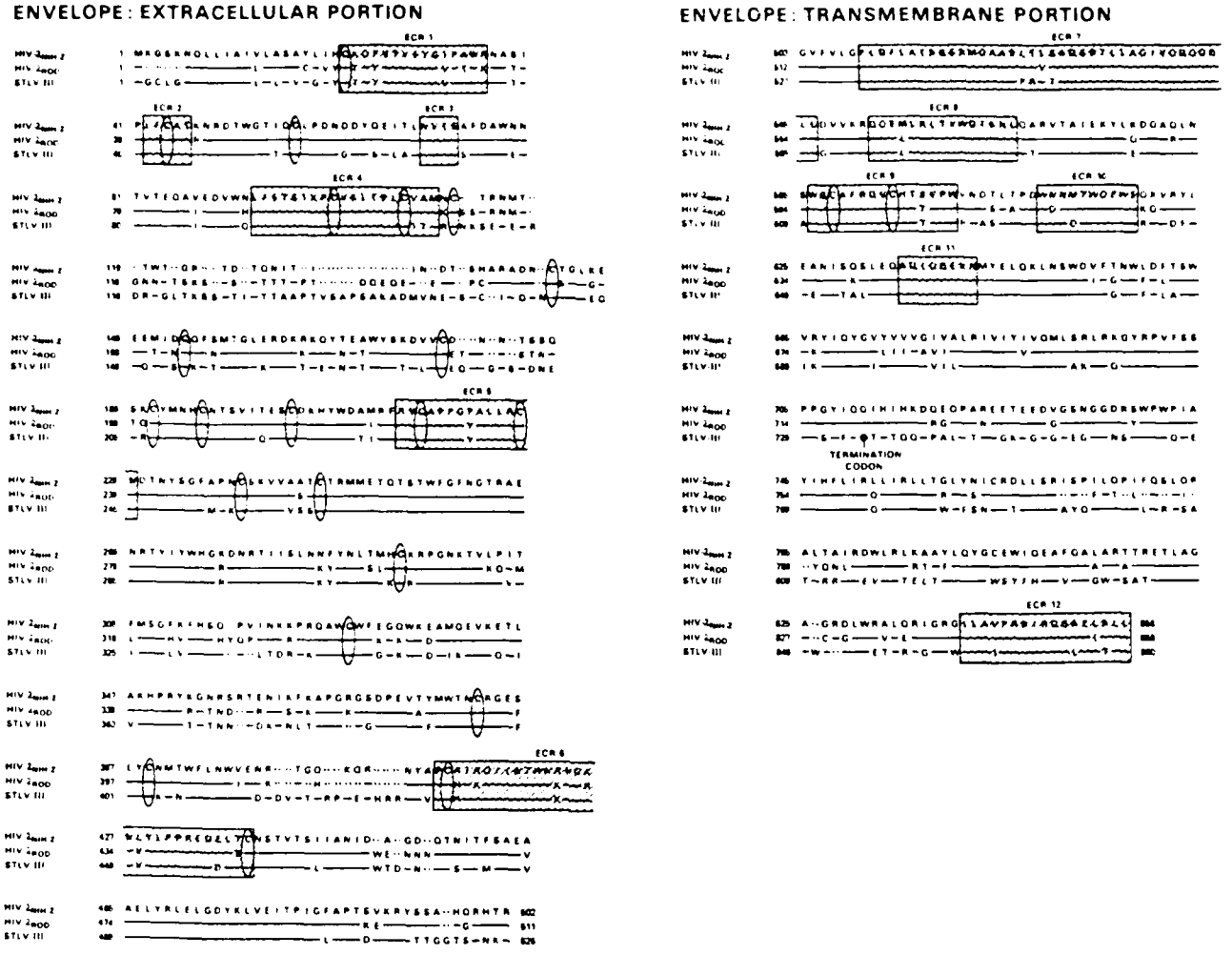



FIGURE 34: **Identification of Variable and Conserved Regions in the Envelope Proteins of SIV and HIV-2.**

This figure represents a comparative analysis of the envelope proteins of HIV-$2_{NIH-Z}$, HIV-$2_{ROD}$ and STLV-III ($SIV_{MAC}$) by amino acid alignment. The left portion of this figure represents the extracellular envelope proteins while the right portion contains the transmembrane portion of the envelope proteins. The amino acid sequence of the HIV-$2_{NIH-Z}$ envelope gene is reported as a line indicating a perfect match in amino acid sequence while the dotted line stands for the lack of the amino acid. The boxes include regions (ECR: envelope conserved regions) which match well and exhibit only conservative amino acid changes with respect to the HTLV-IIIB strain of HIV-1. The dotted ovals indicate highly conserved cysteine residues in both HIV-2 isolates, SIV (STLV-III) and HIV-1 (HTLV-IIIB). The empty ovals indicate cysteine residues that are conserved only in the West African viral isolates and STLV-III. The location of the termination codon in the STLV-III transmembrane envelope protein is indicated.

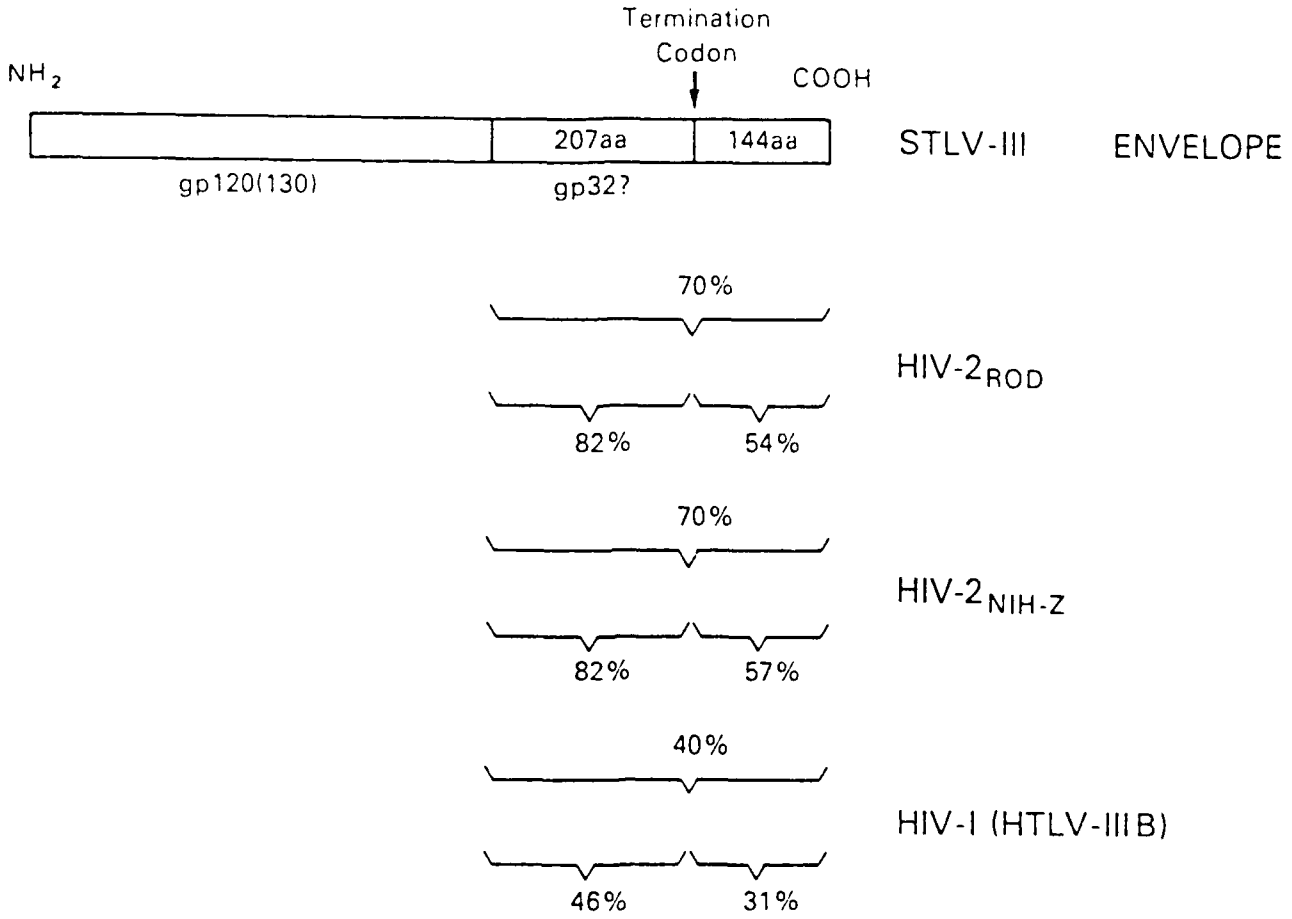



**FIGURE 35: Schematic Representation of the Envelope Proteins of $SIV_{MAC}$ and Percentage of Amino Acid Conservation with other Viral Isolates Before and After the Premature Stop Codon.**

A schematic representation of the transmembrane region of the envelope protein is presented and the overall percentage of homology between $SIV_{MAC}$ and both HIV-2 and HIV-1 is provided. Underneath the line, the homology between each viral transmembrane protein and the SIV transmembrane protein is represented before and after the stop codon.

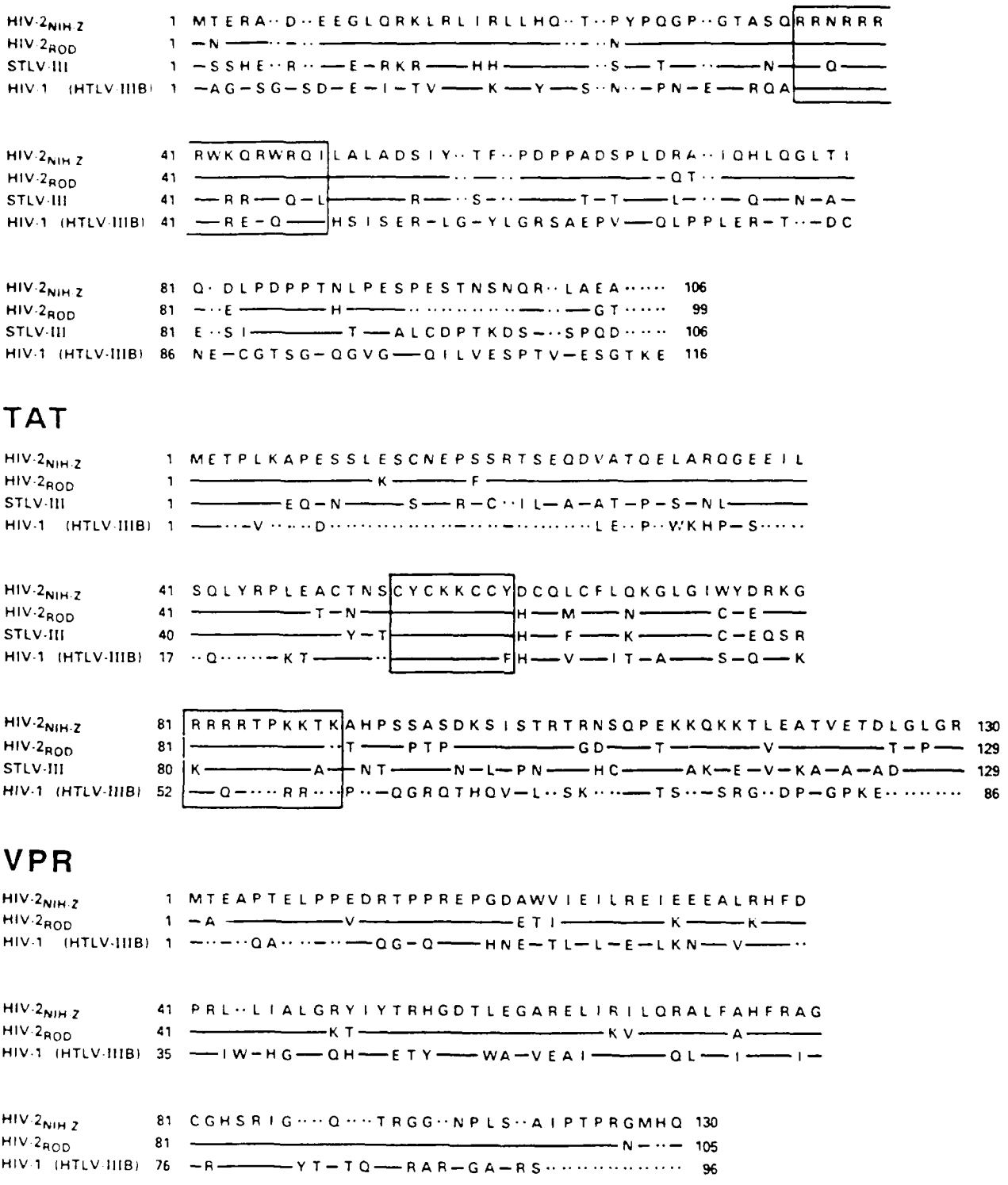
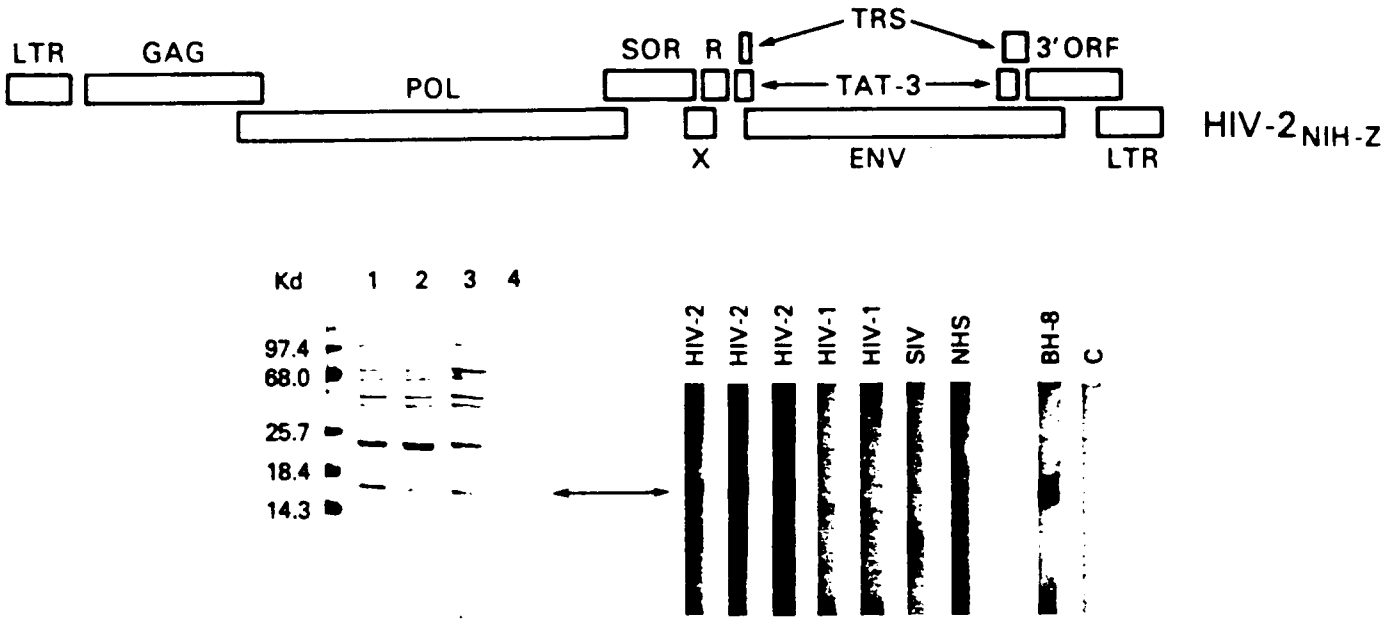



FIGURE 36: Amino Acid Alignment of Other Viral Proteins.
The amino acid sequence of the HIV-$2_{NIH-Z}$ rev, tat and vpr proteins is represented in the top line of each panel. The continuous line represents matching amino acids among the viral isolates. The boxes include regions which are conserved in HIV-1 (HTLV-IIIB).




| Source | N°Tested | N°Positive | %Positive |
|---|---|---|---|
| HIV-2 Infected Humans | 26 | 11 | 42 |
| HIV-1 Infected Humans | 8 | 0 | 0 |
| SIV Infected Macaques | 5 | 0 | 0 |
| Uninfected Humans | 4 | | |
| Total | 44 | | |

**FIGURE 37: Analysis of the HIV-2 vpx Gene.**
The right panel of the figure represents a SDS/PAGE gel of total E. coli proteins stained with Coomassie blue. Lanes 1 to 3 contain protein lysates from E. coli transfected with the vpx (X-orf) of HIV-$2_{NIH-Z}$ in the REV expression vector. As a negative control E. coli were transfected with the REV vector (Lane 4). A specific band with a relative migration corresponding to a molecular weight of approximately 17 Kd is indicated by an arrow. The left part of the figure represents an autoradiogram of a Western blot performed on the total E. coli protein lysate containing the recombinant vpx (X-orf) with human sera from HIV-1 and HIV-2 infected individuals, normal human serum (NHS), monkey serum from an animal infected with SIV, and mouse monoclonal antibodies (BH8) directed against the vector epitopes which are located at the amino terminus of the recombinant protein and a mouse antibody control (C). The arrow indicates that the 17 Kd band reacts with the HIV-2 positive human sera. The recombinant protein for vpx was analyzed by immunoblot using sera from HIV-1 and HIV-2 seropositive humans, SIV seropositive monkeys, and normal human control sera. The 17 Kd band was recognized by 42% of the HIV-2 infected human (table above) and none of the others.

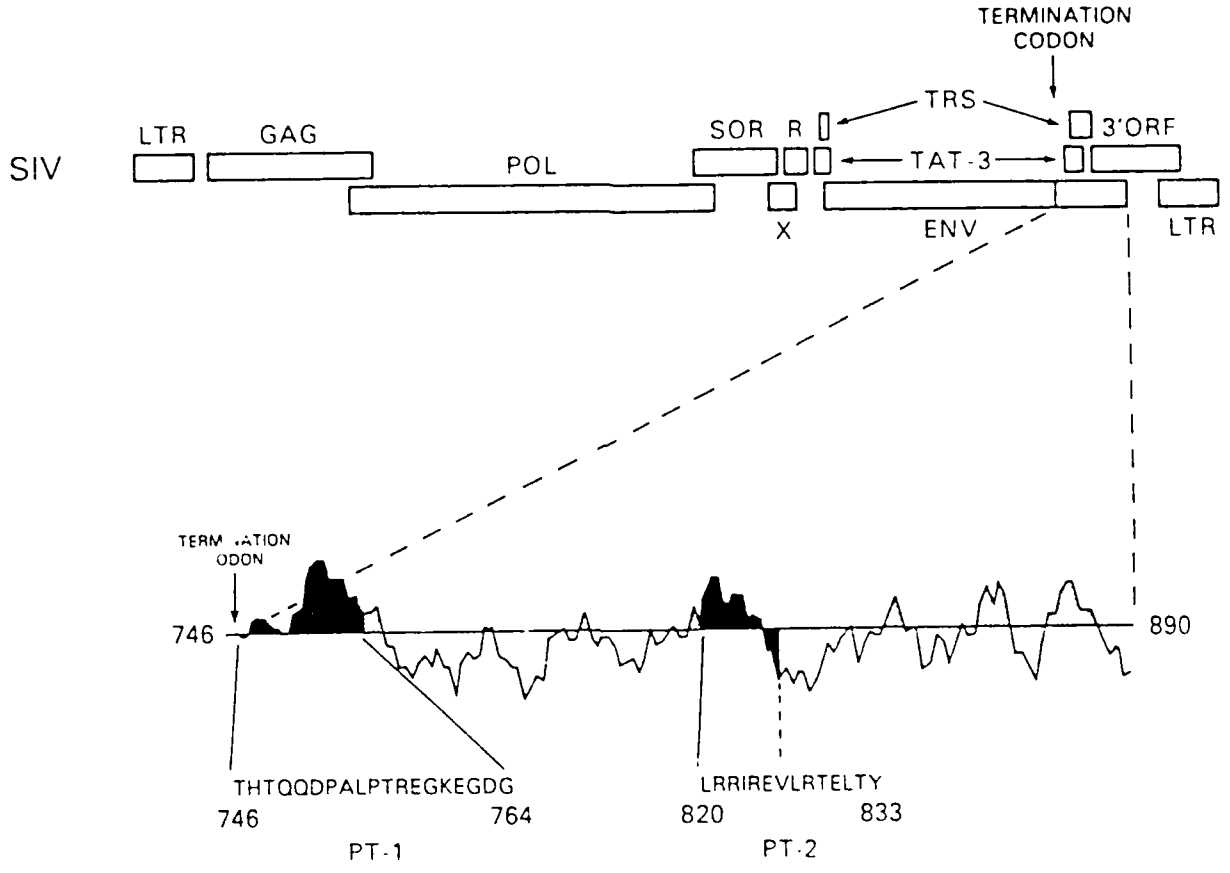



**FIGURE 38: The SIV Genome and the Regions used to make Synthetic Peptides.** The upper part of the figure represents the genome of SIV along with the relative positions of the various genes. The position of the termination codon in the transmembrane portion of the envelope is indicated by the arrow. The lower part of the figure shows the hydropathy profile of the region after the termination codon. The dotted areas are hydrophilic; whereas, the clear areas are hydrophobic. The darken areas are regions used for PT-1 and PT-2 peptides.

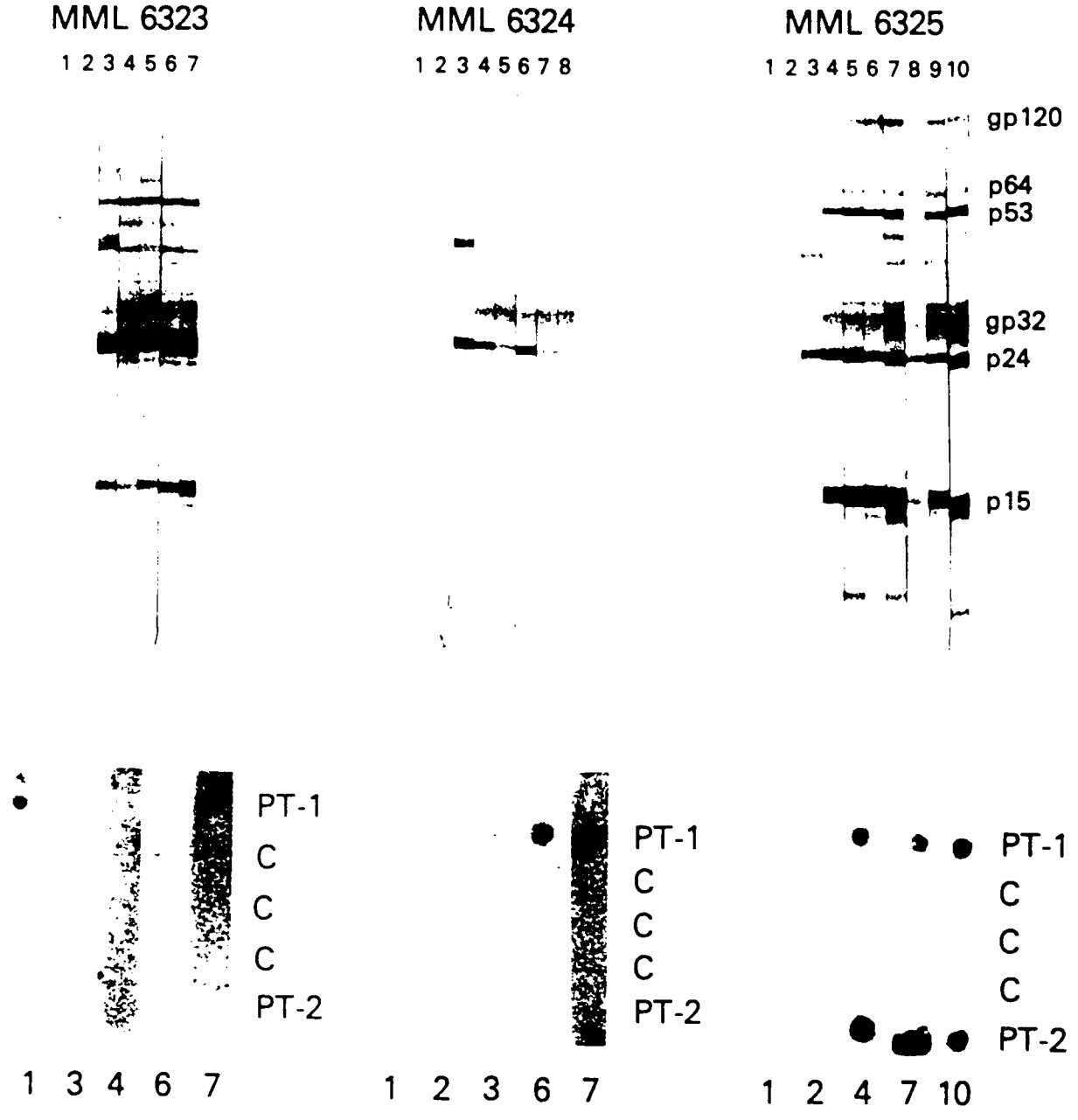



**FIGURE 39: Western Blot Analysis of Sera Obtained from Rhesus Macaques.** Three rhesus macaques were inoculated with SIV (MML 6323, MML 6324, and MML 6325). In the upper part of the figure the sera were reacted with nitrocellulose strips containing total SIV proteins. Viral proteins were obtained from the same SIV strain that was used to inoculate the animals. The lower portion of the figure shows results obtained when sera from the same three animals were reacted with the synthetic peptides PT-1 and PT-2 (1 μg) and the control peptide (1, 2, and 3 μg). The numbers on the abscissa indicate the date that the sera were obtained following infection as expressed in months.

PT-1

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SIV | T | H | T | Q | Q | D | P | A | L | P | T | K | E | G | K | K | G | D | G |
| | . | | . | . | . | | . | . | . | | . | . | | . | . | . | . | | . |
| HIV-$2_{NIH-Z}$ | I | H | I | H | K | D | Q | E | Q | P | A | R | E | E | T | E | E | D | V |
| | . | | . | . | . | | . | . | . | | . | . | | . | . | . | . | | |
| HIV-$2_{ROD}$ | I | H | I | H | K | D | R | F | Q | P | A | N | E | E | T | E | E | D | G |
| | . | | . | . | . | | . | . | . | | . | . | | . | . | . | . | | . |
| HIV-$2_{ISY}$ | I | H | I | H | K | D | W | E | Q | P | D | R | E | E | T | E | E | D | V |

PT-2

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SIV | L | R | R | I | R | E | V | L | R | L | E | L | T | Y |
| | | . | . | | | . | . | | | | . | . | . | |
| HIV-$2_{NIH-Z}$ | L | T | A | I | R | D | W | L | R | L | K | A | A | Y |
| | | . | . | . | | . | . | | | | | . | . | . |
| HIV-$2_{ROD}$ | Y | Q | N | L | R | D | W | L | R | L | R | T | A | F |
| | | . | . | . | | . | . | | | | . | . | . | |
| HIV-$2_{ISY}$ | L | Q | P | L | R | D | W | L | R | L | K | A | A | Y |

**FIGURE 40: Comparison of Amino Acids Among Human HIV-2 Isolates.** Amino acid alignment of the SIV sequences which correspond to PT-1 and PT-2 with the equivalent sequence of three HIV-2 isolates. The dots indicate the diversity in amino acid composition.

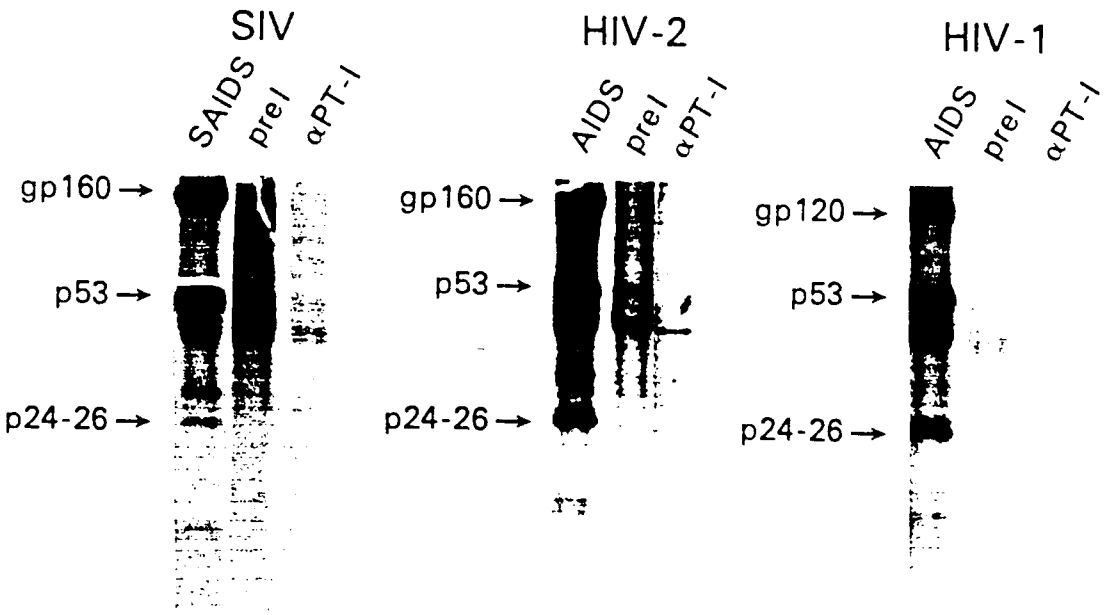



**FIGURE 41: Reactivity of Rabbit Antiserum Against the Synthetic Peptide PT-1.** Immunoprecipitation of metabolically labeled total cellular proteins from the SIV-infected human T-cell line, HUT 78. SAIDS -- serum from macaque 6325 (bleed 10) which was infected with SIV; AIDS -- serum from patients with acquired immunodeficiency syndrome; preI -- rabbit preimmune serum; anti-PT-1 -- immune serum raised against the synthetic peptide PT-1.

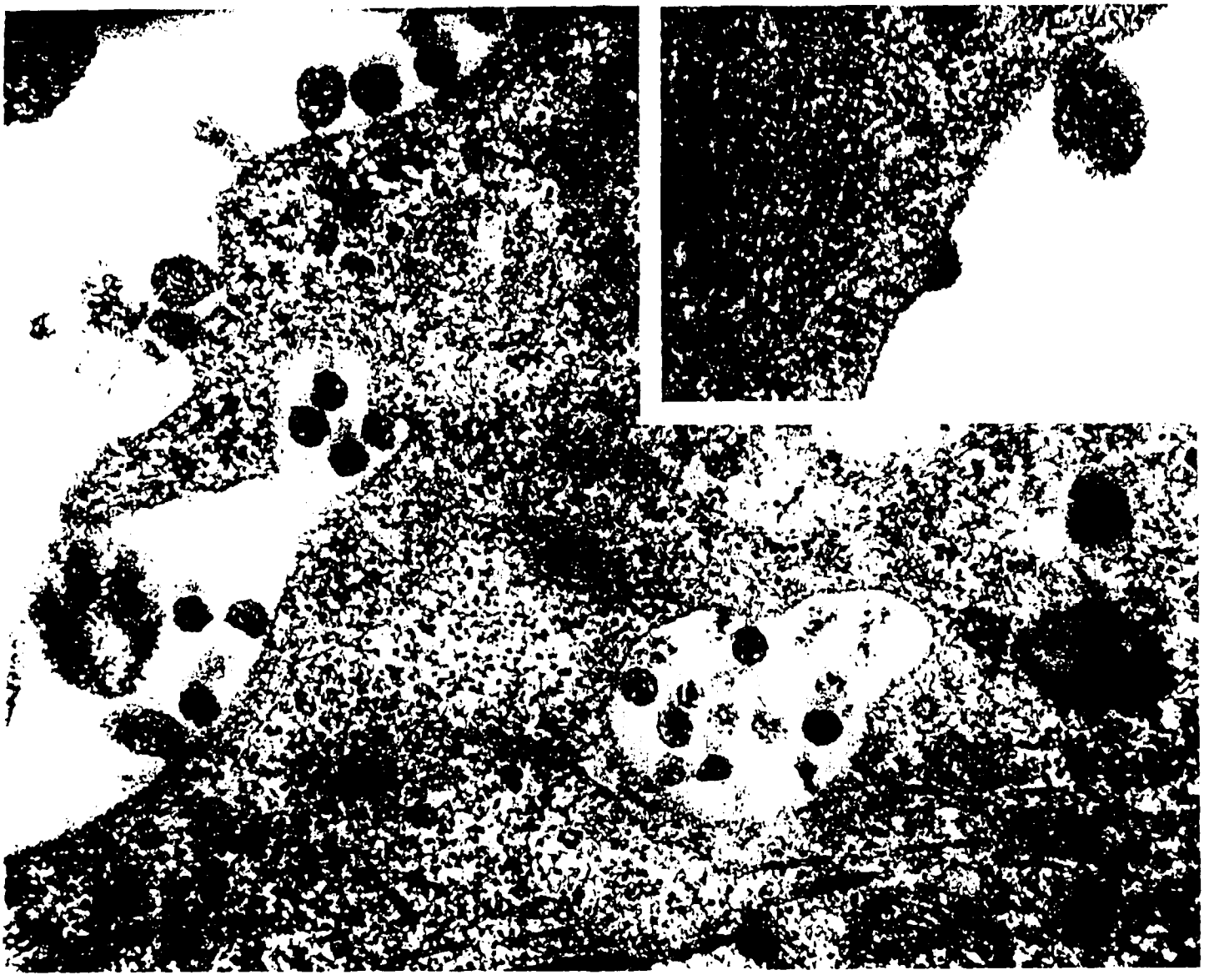


**FIGURE 42: Electron Micrograph of the HIV-$2_{ISY}$.**
The inset includes a section in which a budding viral particle from a HUT78 cell can be detected. Several mature virions with dense cylindrical or round core can be seen in the remainder of the figure.

# CYTOPATIC EFFECT OF HUMAN IMMUNODEFICIENCY VIRUSES TYPE 2

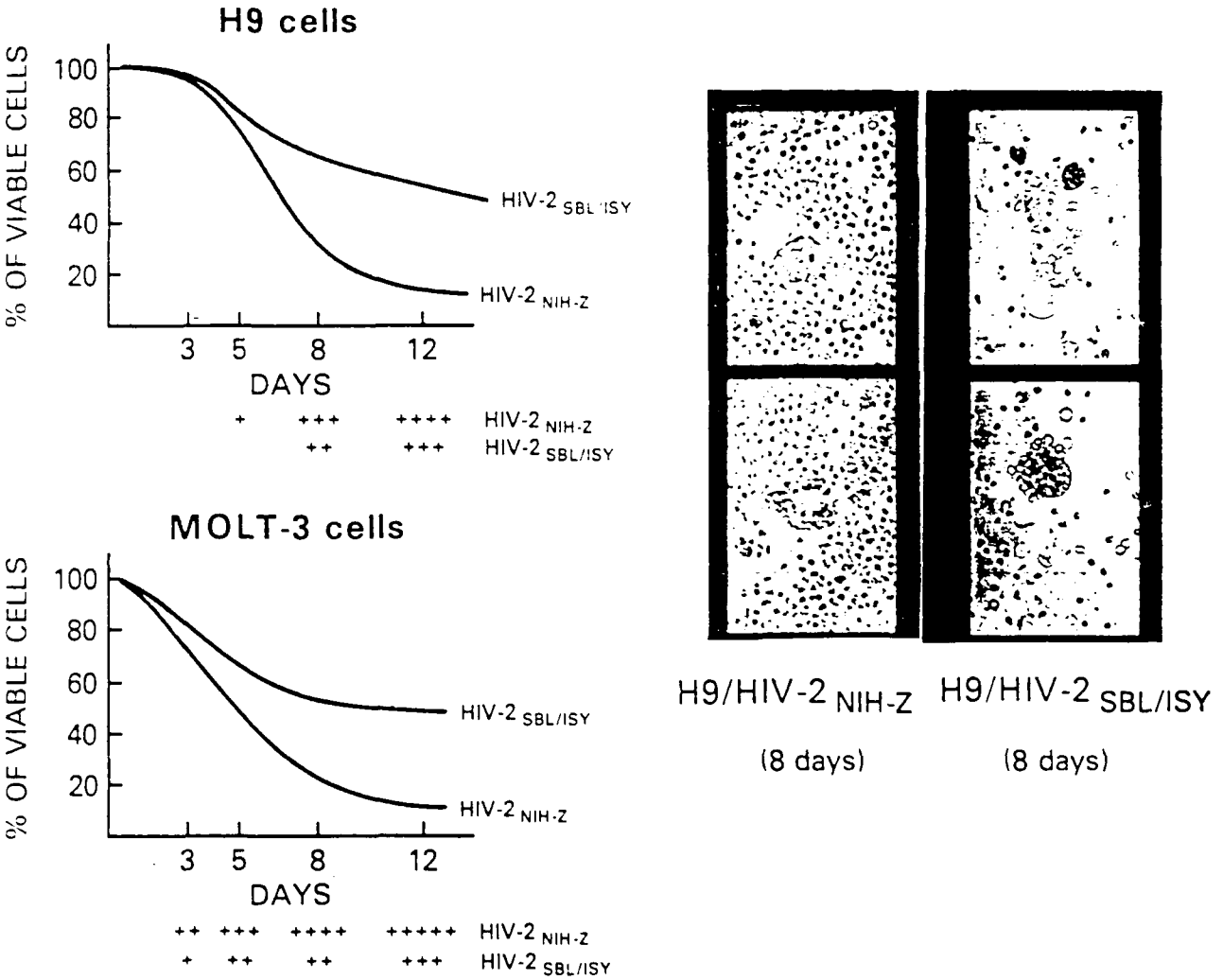



**FIGURE 43: Cytopathic Effect of Human Immunodeficiency Viruses Type 2.** The left portion of the figure graphically represents the reduction in the number of viable cells in the culture after infection with HIV-$2_{NIH-Z}$ and HIV-$2_{ISY}$ isolates. The + symbol represents the relative scale of syncytia seen in cell culture at 3, 5, 8, and 12 days. An example of the size of syncytia seen at day 8 for both viral isolates is shown on the right side of the figure.

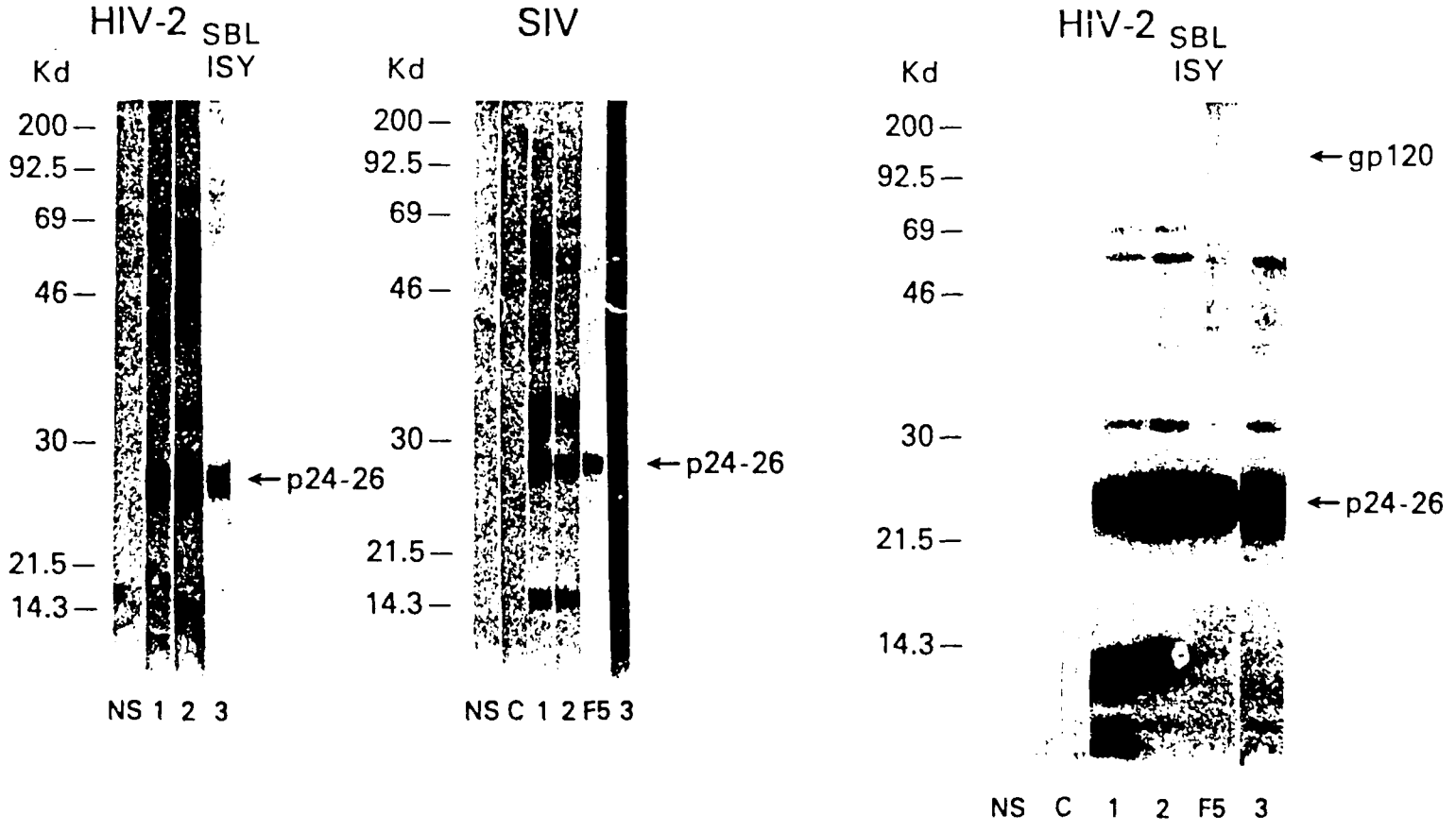



**FIGURE 44: Western Blot Analysis of Viral Proteins.**
The left and central panels represent the Western blot analyses of total viral proteins obtained from disrupted $HIV_{ISY}$ and SIV virions, respectively. The right panel represents an immuno-precipitation of metabolically labeled HIV-$2_{ISY}$ virions. NS = normal human serum; 1 = serum from a macaque experimentally infected with SIV, 2 and 3 = sera from human infected with HIV-2, c = control mouse ascite, F5 = mouse monoclonal antibody directed against the p24 of SIV. The molecular weight of the proteins was calculated with respect to the migration of standard markers from BRL.

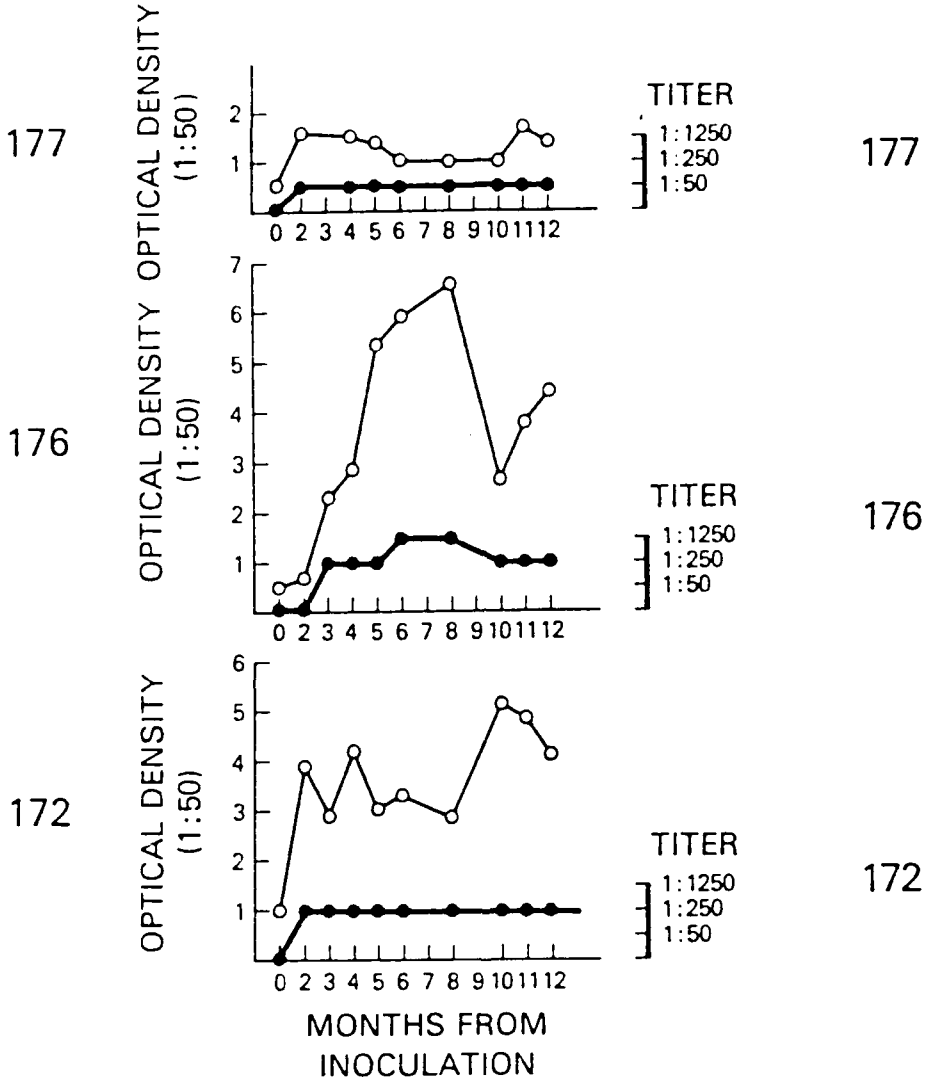



**FIGURE 45: Detection of Antibodies in Infected Rhesus Macaques by ELISA.** Virions from HIV-$2_{NIH-Z}$ were purified on a sucrose gradient, lysed and bound to a microtiter plate. The plates were reacted with varying dilutions of the monkey sera and the immunocomplexes were detected using goat antihuman antibodies conjugated to horse radish peroxidase for color development. The left Y-axis indicates the optical density obtained with a 1:50 dilution of sera obtained from animals #177, 176, and 172. The right Y-axis is the actual titer of the antibodies. The X-axis indicates the months after inoculation. Animals #177 and 176 were infected with HIV-$2_{ISY}$ while animal #172 was infected with HIV-$2_{NIH-Z}$.

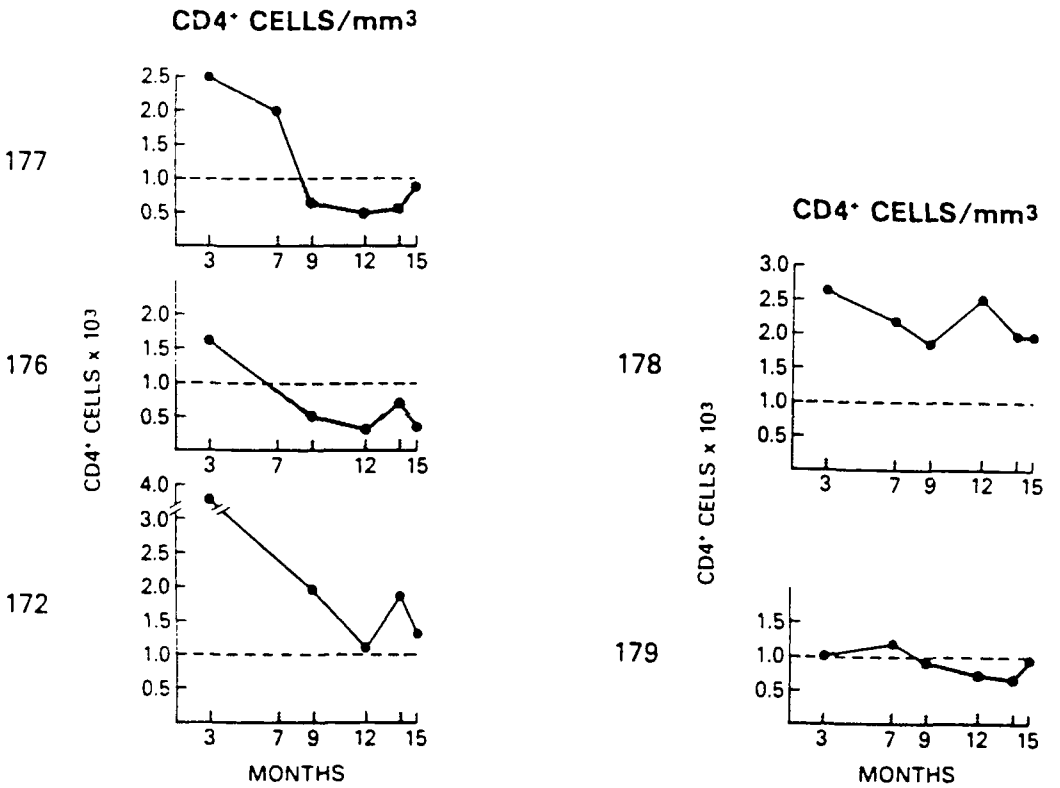



**FIGURE 46: Expression of Surface Membrane CD4 and CD8 Antigens.** Direct immunofluorescence assay on live cells using phycoerythrin-conjugated Leu 3a (anti-CD4) and fluorescein-isothyocyanate-conjugated Leu 2a (anti-CD8) monoclonal antibodies was used to assess the expression of CD4 and CD8 antigens. Animals #177 and #176 were inoculated with HIV-$2_{ISY}$. Animal #172 was inoculated with HIV-$2_{NIH-Z}$. Animals #178 and #179 were inoculated with saline and represent negative control standards.

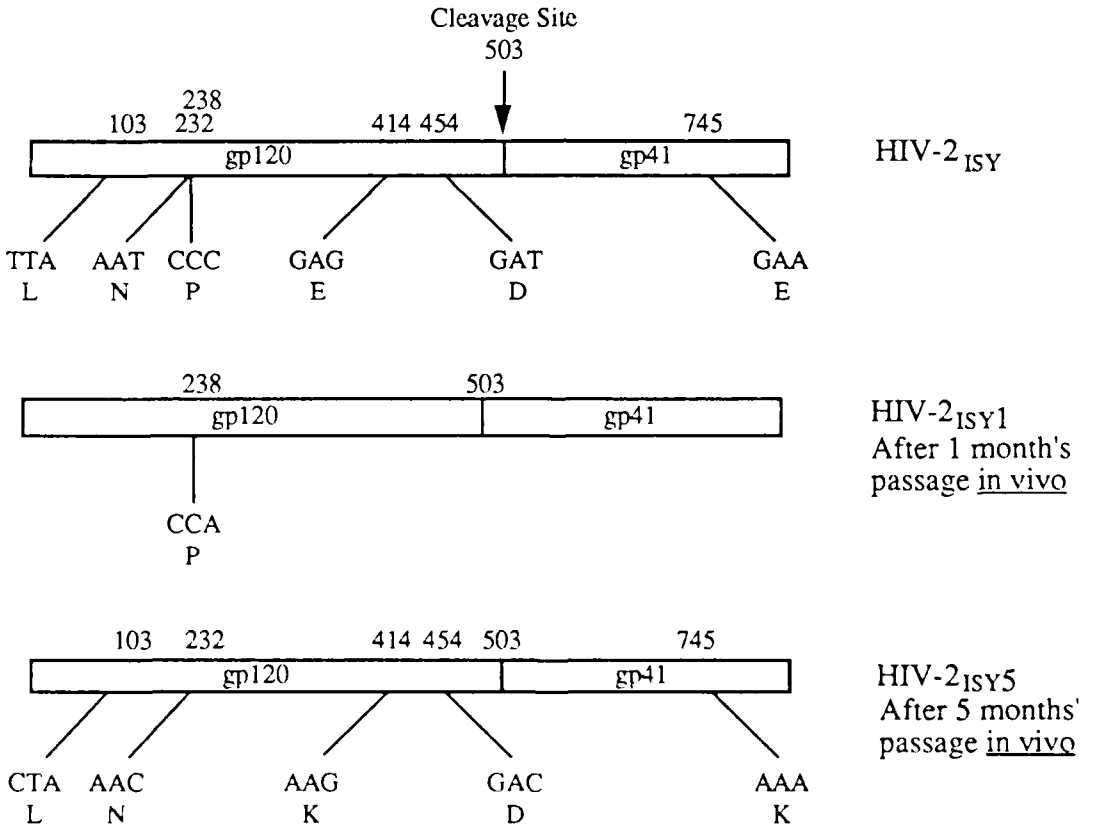



**FIGURE 47. HIV-2 Envelope Gene Heterogeneity.**
Location of nucleotide and amino acid changes in HIV-$2_{ISY}$ after 1 and 5 months of infection in Rhesus macaques. The single letter amino acid codes were used as indicated: L=Leucine, P=proline, E=glutamic acid, K=Lysine, N=Asparagine, D=Aspartic acid.

| New Gene Name | Previous Name | MW of Protein | Known Function |
|---|---|---|---|
| tat | tat-3, TA | p14 | Transactivator of all viral proteins |
| rev (Transregulator of expression of virion proteins) | art, trs | p19 | Regulates expression of virion proteins |
| vif (Virion infectivity protein) | sor, A, P', Q | p23 | Determines virus infectivity |
| nef (retrovirus inhibitory factor) | 3'orf, B, E', F | p27 | Reduces virus expression, GTP-binding |
| vpr (R) | R | ? | Unknown |
| vpu (U) | U | p16 | Unknown |

**TABLE 1: Nomenclature of HIV-1 Accessory Genes.**

| Sample | Supernatants Removed from Molt3 Cultures and Concentrated x 100 | | Infectivity | Trans-activation in H9 Cells | Trans-activation in Cos-1 Cells |
|---|---|---|---|---|---|
| | RT Activity dpm | Virus Concentration (particles per ml) | | Mean Conversion (%) | Mean Conversion (%) |
| X | 138,857 | $3.7 \times 10^{10}$ | + | 40.5 ± 3.9 | 56.1 ± 19.9 |
| ΔS | 137,743 | $2.6 \times 10^{10}$ | - | 32.0 ± 6.9 | 67.5 ± 28.9 |
| 3.3 | 120,262 | $2.9 \times 10^{10}$ | - | 43.0 ± 8.9 | 62.0 ± 17.5 |
| 6.9 | 74,794 | $2.4 \times 10^{10}$ | - | 54.3 ± 23.9 | 37.5 ± 15.5 |
| 153 | 61,383 | $3.3 \times 10^{10}$ | - | 29.8 ± 2.5 | 47.0 ± 25.7 |

**TABLE 2: Infectivity and Properties of vif Mutant Viruses of HIV-1.**
Molt 3 were infected with HIV-1 by coculturing with Cos-1 cells transfected with X, ΔS, 3.3, 6.9 and 153 virus. The presence of virus was assayed by Reverse Transcriptase activity. Viral particles were counted by electronmicroscopy. Transactivation activity was measured by co-transfecting the plasmid along with pC15CAT into H9 or Cos-1 cells. The resulting CAT activity was determined in each case.

| | Day 0 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| ΔS | | | | | |
| $1x10^5$ | 10% | 30% | 28% | 16.0% | 2.0% |
| $2x10^5$ | 20% | 50% | 23% | 20.0% | 2.0% |
| HXB2D | | | | | |
| $1x10^5$ | 10% | 30% | 2.00% | 3.5% | 9.0% |
| $2x10^5$ | 20% | 40% | 8.75% | 25.0% | 35.0% |

**TABLE 3: Kinetics of vif Mutant Transmission.**
Mitomycin-C treated Molt-3 cells were infected with virus from Cos-1 transfected cells ($1 \times 10^5$ or $2 \times 10^5$). The percentage of cells expressing HIV p24 was examined at weekly intervals by the immunofluorescence assay.

Cell-Associated Versus Cell-Free HIV-1 Neutralization
Results Using Wild-Type and vif Defective Clones
Difference between Cell-Free and Cell-Associated Titers

| Sera ----- / Virus | HS01 | HS02 | HS03 | HS04 | HS05 | HS06 | HS07 | HS08 | HSB09 | HS10 | HS11 | HS12 | HS13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Reciprocal Geometric Mean 50% Neutralization Titer | | | | | | | | | | | | |
| III | 16 | 1 | 16 | 1 | 16 | 256 | 1 | 1 | 16 | 16 | 64 | 1024 | 1 |
| HXB2D | 4 | 1 | 64 | 1 | 1 | 64 | 16 | 1 | 4 | 4 | 64 | 4096 | 1 |
| DA4/Xgpt | 80 | 320 | 20 | 20 | 20 | 5 | 1 | 1 | 20 | 20 | 5 | 20 | 5 |
| AC4/3.3 | 1 | 20 | 20 | 5 | 1 | 1 | 1 | 1 | 20 | 20 | 5 | 5 | 1 |
| AEC1/153 | 5 | 80 | 5 | 5 | 1 | 5 | 1 | 1 | 20 | 80 | 5 | 5 | 1 |

| | HS14 | HS15 | HS16 | HS17 | HS18 | HS19 | HS20 | HS21 | HSB22 | HS23 | HS24 | HS25 | HS26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| III | 1024 | 64 | 4 | 4096 | 256 | 1 | 256 | 4 | 1 | 64 | 1 | 4096 | 4096 |
| HXB2D | 64 | 16 | 16 | 16 | 64 | 1 | 64 | 4 | 1 | 16 | 16 | 64 | 64 |
| DA4/Xgpt | 5 | 5 | 20 | 1 | 5 | 5 | 1 | 1 | 20 | 20 | 20 | 80 | 1 |
| AC4/3.3 | 1 | 5 | 5 | 1 | 5 | 1 | 1 | 1 | 1 | 20 | 20 | 1 | 1 |
| AEC1/153 | 1 | 1 | 20 | 1 | 1 | 1 | 1 | 1 | 5 | 80 | 5 | 1 | 1 |

**TABLE 4: Cell-Associated Versus Cell-Free Neutralization of Wild Type vif Defective Mutants of HIV-1.**
H9 cells were infected with 4 times the TCID50 of HXB2D, HTLV-IIIB (III) virus or cocultured with cloned cell lines infected with vif mutant viruses. The cells were treated with serial dilutions of various human sera positive for HIV to examine the ability of these sera to neutralize virus transmission. Expression of virus was examined at 2 weeks post infected by immunofluorescence with anti HIV-1 p24. The dilution of serum which gave a 50% reduction in virus titer is expressed as a reciprocal geometric mean titer.

| | Mode of Transmission | | | |
|---|---|---|---|---|
| | Cell-to-Cell | | Cell-Free | |
| Virus | AC4/AEC1 | DA4/Xgpt | HXB2D | HTLV-IIIB |
| | Geometric Mean Titers of WR Stage 2 Patients of Given Status | | | |
| Progression | 1:2 | 1:11 | 1:32 | 1:1024 |
| No Progression | 1:2 | 1:4 | 1:11 | 1:24 |

**TABLE 5: Cell-Associated Versus Cell-Free HIV-1 Neutralization as a Function of Progression to AIDS.**
Sera from a patient who progressed to AIDS and from one who had not progressed within 4 years were incubated with wild type or mutant viruses. Serial dilutions of the sera were used to infect H9 cells. The results are expressed as the dilution which gave a 50% reduction of virus titer as determined by immunofluorescence with anti HIV-1 p24.

| | Mode of Transmission | | | |
|---|---|---|---|---|
| | Cell-to-Cell | | Cell-Free | |
| Virus | AC4/AEC1 | DA4/Xgpt | HXB2D | HTLV-IIIB |
| | Geometric Mean Titers of Patients of Given Status | | | |
| WR Stage 2 | 1:2 | 1:6 | 1:16 | 1:73 |
| WR Stage 6 | 1:7 | 1:25 | 1:3 | 1:2 |

**TABLE 6: Cell-Associated Versus Cell-Free HIV-1 Neutralization as a Function of Disease Stage.**
Sera from a patient at Walter Reed Stage 2 and from the same patient at Walter Reed Stage 6 were examined for their ability to neutralize virus transmission by the cell-to-cell versus the cell-free route.

| Construct | AA Changes in env | Replication RT/Cos-1 (Days) | Transmission H9 gag+ | Molt-3 gag+ |
|---|---|---|---|---|
| (Parental Clone - No Deletion) | | | | |
| HXB2D | -00 +00 | 2+ | +++ | +++ |
| (Mutants with Deletions in nef only) | | | | |
| X330 | -00 +00 | 7+ | +++ | +++ |
| (Mutants with Deletions in gp41 and nef) | | | | |
| X329 | -00 +47 | 7+ | ++ | ++ |
| X206 | -04 +59 | 2+ | +++ | +++ |
| X10-1 | -05 +15 | 2+ | +++ | +++ |
| X9-3 | -05 +76 | 7+ | +++ | +++ |
| X369 | -06 +02 | 7+ | +++ | + |
| X295 | -14 +02 | 14+ | +++ | ++ |
| X372 | -15 +04 | 14+ | +++ | + |
| X429 | -15 +04 | 14+ | ++ | - |
| X269 | -17 +02 | 14+ | ++ | - |
| X468 | -33 +00 | 14+ | ++ | - |
| X362 | -37 +18 | 14+ | ++ | - |
| (Mutants with Deletions in the LTR/PPT as well as gp41 & nef) | | | | |
| X358 | -06 +03 | 7+ | - | - |
| X318 | -06 +04 | 7+ | - | - |
| X204 | -06 +27 | 7+ | - | - |
| X312 | -14 +74 | 7+ | - | - |
| X192 | -17 +11 | 7+ | - | - |
| X189 | -41 +00 | 7+ | - | - |
| X274 | -42 +02 | 7+ | - | - |
| X194 | -76 +09 | 7+ | - | - |
| (Mutants with Deletions in tat/rev as well as above) | | | | |
| X360 | -87 +32 | - | - | - |
| X327 | -117+20 | - | - | - |

Key: + < 20% positive in culture after four weeks.
++ > 20% positive in culture in two weeks or less.
+++ > 20% positive in culture in one week or less.

**TABLE 7: Replication and Transmission of C-Terminal env Deletion Mutants.**
Cos-1 cells were transfected with pHXB2D (plasmid containing the HIV-1 genome) or deletion mutants of this plasmid. Virus replication was assayed by testing for reverse transcriptase activity in the spent supernatant of transfected cells at 2 days to 2 weeks post transfection. Viral transmission was assayed by cocultivating polybrene treated H9 or Molt-3 cells with transfected cells for two days, separating the H9 or Molt-3 cells, and monitoring for the production of gag p24 protein by immunofluoresence of cells fixed in acetone up to 4 weeks.

| Virus Clone | Changes in gp41 | Particle Count $10^{11}$/ml | RT pM/ml/hr | H9 | | MOLT-3 | |
|---|---|---|---|---|---|---|---|
| | | | | $TCID_{50}$+ | MOI++ | $TCID_{50}$+ | MOI++ |
| HXB2D | - 0 + 0 | 1.8 | $8.0 \times 10^4$ | 2,435 | 18 | 2,048 | 22 |
| X10-1 | - 5 + 15 | 1.0 | $1.3 \times 10^4$ | 362 | 70 | 181 | 140 |
| X9-3 | - 5 + 70 | 2.5 | $1.9 \times 10^4$ | 1,878 | 31 | 861 | 72 |
| X295 | - 14 + 2 | 2.8 | $3.7 \times 10^4$ | 235 | 300 | 49 | 1,400 |
| X429 | - 15 + 4 | 2.8 | $7.6 \times 10^4$ | 29 | 2,700 | 87 | 800 |
| X269 | - 17 + 2 | 2.8 | $8.1 \times 10^4$ | 3,158 | 22 | 558 | 110 |
| X468 | - 33 + 0 | 1.9 | $4.4 \times 10^4$ | 49 | 910 | 54 | 870 |
| X362 | - 37 + 18 | 0.4 | $2.2 \times 10^4$ | 4,467 | 3 | 790 | 13 |

+ Tissue Culture Infectious Dose - 50% values calculated by the method of Reed and Muench from 8 parallel dilutions: values shown represent the reciprocal of that dilution of 20 µl of virus preparation which produced detectable infection in half the cultures (the $TCID_{50}$/ml may be calculated by multiplying the figures shown by 50).

++ Number of virions/cell required to produce 50% infection (5 µl preparation, $2 \times 10^4$ cells).

**TABLE 8: Cell-Free Transmission of C-Terminal <u>env</u> Deletion Mutants.**
Results of titrations and virus preparation characteristics are shown for single virus preparations of the selected clones indicated. Particle counts were performed by negative-contrast transmission electron microscopy. Reverse transcriptase values represent individual determinations.

| Clone | Amino Acids Changed in gp41[a] | Nucleotides Deleted | Other Genes Affected[b] | RT[c] Production | Infectivity[d] H9[e] | Infectivity[d] MOLT-3[f] |
|---|---|---|---|---|---|---|
| HXB2gpt | -0+0 | None | None | + | + | + |
| X329 | -0+47 | 8370-8601 | nef | + | + | + |
| X206 | -4+59 | 8357-8564 | nef | + | + | + |
| X10-1 | -5+15 | 8456-8655 | nef | + | + | + |
| X9-3 | -5+70 | 8456-8518 | nef | + | + | + |
| X358 | -6+3 | 8351-8728 | nef/LTR | + | - | - |
| X295 | -14+2 | 8328-8627 | nef | + | + | + |
| X429 | -15+4 | 8326-8601 | nef | + | + | - |
| X269 | -17+2 | 8320-8628 | nef | + | + | - |
| X468 | -33+0 | 8270-8636 | nef | + | + | - |
| X362 | -37+18 | 8259-8579 | nef | + | + | - |
| X274 | -42+2 | 8244-8736 | nef/LTR | + | - | - |
| X194 | -76+9 | 8142-8711 | nef/LTR | + | - | - |
| X360 | -187+32 | 7809-8537 | tat/rev/nef/LTR | - | - | - |
| X327 | -177+20 | 7839-8573 | tat/rev/nef/LTR | - | - | - |
| pSV2neo | N/A | N/A | N/A | - | - | - |

**TABLE 9: Summary of Properties of C-Terminal env Deletion Mutants.**

[a] First number denotes number of amino acides missing from expected sequence of HXB2. Second number denotes predicted number of additional amino acids until a random stop codon is reached after Bal 31 digestion and self-ligation was performed to construct mutants shown.

[b] Additional reading frames affected by Bal 31 deletions.

[c] Reverse transcriptase activity.

[d] Ability of clones to infect H9 and MOLT-3 lymphocyte lines is shown in the last two columns. Concurrence was seen in at least triplicate cocultures.

[e] Able to infect H9 cells by coculture.

[f] Able to infect MOLT-3 cells by coculture.

| VIRUS | % of Cells Killed ATH8 (H9) | ATH8 (Molt3) | HPB-ALL (H9) | HPB-ALL (Molt3) |
|---|---|---|---|---|
| None | 0 | 0 | 0 | 0 |
| HXB2D | 100 | 100 | 100 | 100 |
| X10-1 | 0 | 4 | 0 | 31 |
| X9-3 | 0 | 0 | 25 | 31 |
| X295 | 0 | 65 | 0 | 50 |
| X362 | 0 | 0 | 0 | 80 |

**TABLE 10: Cytopathic Effects of Cell-Associated Transmission of C-Terminal <u>env</u> Deletion Mutants.**

Infected cells ($5 \times 10^4$) of mutant virus propagated in H9 or Molt cells were washed and added to $1 \times 10^6$ polybrene treated ATH8 or HPB-ALL cells. Cells were incubated in RPMI-20% fetal bovine serum and viability was assessed at one week by exclusion dye trypan blue.

| Mutant | p24 Antigen (pg) | HIV-1 RNA (cpm) | RNA/Antigen cpm/pg |
|---|---|---|---|
| #3 | 285 | 319 | 1.12 |
| #79 | 345 | 352 | 1.02 |
| HXB2-gpt | 372 | 1,215 | 3.27 |
| H9/HIV-1 (positive control) | 360 | 967 | 2.69 |
| COS-1 (negative control) | --- | 45 | ---- |

**TABLE 11: Ratios of RNA to Antigen in Virus Produced in COS-1 Cells Transfected by Packaging Mutants.**
COS-1 Cells were transfected with mutant viruses and viral particles were collected from the supernatant at 2 days post transfection. The amount of p24 antigen in the virus was determined by antigen capture. Virus specific RNA content was determined by spot blot hybridization with $^{32}P$ labeled HIV-1 plasmid DNA. The ratio of RNA to antigen is expressed as cpm hybridized per pg p24.

| Mutant (Dilution) | p24Ag pg (1/6,400) | p24Ag Total pg | RNA cpm (1/500) | RNA Total cpm | RNA/Ag cpm/ng |
|---|---|---|---|---|---|
| #3 | .063 | 403 | 52 | 26,000 | 64.5 |
| #79 | .061 | 390 | 17 | 8,500 | 21.8 |
| #82 | .066 | 422 | 101 | 50,500 | 119.7 |
| HXB-2gpt | .074 | 473 | 143 | 71,500 | 151.2 |
| H9-HIV | .159 | 1018 | 286 | 143,000 | 140.5 |

**TABLE 12. Ratio of RNA to p24 Antigen in Cell-Free Virions of Packaging Mutant Virions.**
Stable strains of mutant virus were propagated in H9 cells. The virions were collected from cell-free supernatants of infected cells. The p24 antigen content and viral RNA content were determined as in Figures 10 and 11.

| Plasmid | Description | 20 Minute Assay H9/HIV Exp. 2 | 15 Hr. Assay H9 Exp. 2 | 15 Hr. Assay H9 Exp. 3 |
|---|---|---|---|---|
| SVO | Negative Control | 0.0 | 0.0 | 0.00 |
| SV2 | Positive Control | 2.6** | 6.7 ± 1.0 | 4.60 ± 1.5 |
| RSV | Positive Control | 18.8 ± 4.8 | 48.4 ± 3.41 | 55.80 ± 3.1 |
| C15CAT | HIV-1 LTR-CAT | 20.8** | 0.1 ± 0.04 | ND |
| CD12CAT | HIV-1 LTR-CAT | 88.8 ± 10.9 | 0.7 ± 0.05 | 0.70 ± 0.080 |
| VHHCAT | HIV-1 LTR-CAT | 92.7 ± 1.3 | ND | 2.40 ± 0.34 |
| -65 | del. -65 from CAP | 32.1** | 0.09 ± 0.03 | 0.00 |
| -48 | del. -48 from CAP | 0.0 | ND | 0.00 |
| -65E2 | 5'-3' -105 to -80 | 99.1 ± 0.1 | ND | 2.70 ± 0.53 |
| -65E5 | 3'-5' -80 to -104 | 99.2 ± 0.1 | ND | 0.85 ± 0.31 |
| -48E9 | 5'-3' -105 to -80 | 26.7** | ND | 0.21 ± 0.061 |
| -48E14 | 5'-3' -105 to -79 | 22.4** | ND | 0.18 ± 0.006 |
| -48E8 | 3'-5' -80 to -104 | 53.8 ± 7.9 | ND | 0.27 ± 0.093 |
| -117 | del. -117 from CAP | 97.5 ± 0.8 | 0.4 ± 0.07 | 0.66 ± 0.14 |
| -117 Δ BS | del. Bgl II to SstI | 0.13 ± 0.02 | 0.2 ± 0.09 | ND |
| -117 Δ S | del. 4 bp at SstI | 1.2 ± 0.3 | 16.7** | ND |

**Transfections done in duplicate only.
NDNot Done.

**TABLE 13: CAT Assay Results in T Cells (H9) and HIV-1 Infected T Cells (H9/HIV).**

Percent conversion of $^{14}$C-chloramphenicol to acetylated metabolites using lysates of cells transiently transfected with the indicated plasmids. Cells used were H9, an uninfected T-cell line and H9/HIV cells, the same cell line productively infected with HIV-1. Transfections were done in triplicate for values showing standard deviations.

| Stimulant | Relative CAT Activity |
|---|---|
| Medium Alone | 1.0 |
| Ionomycin | 1.7 |
| PHA | 5.6 |
| PMA | 8.2 |
| tat-III | 378.0 |
| Ionomycin + tat-III | 805.0 |
| PHA + tat-III | 3,275.0 |

**TABLE 14. The Multiplicative Effects of T-Cell Mitogens and tat-III on HIV LTR-CAT Expression.**
The relative amount of CAT expression in Jurkat cells transfected with an HIV LTR-CAT expression vector was measured following incubation with a series of mitogens or mitogens plus cotransfection with an expression plasmid for tat-III. The results are expressed as fold stimulation relative to basal CAT levels with no stimulation.

| | - - Retrovirus Strain - - | | |
|---|---|---|---|
| | HIV-$2_{NIH-Z}$ | HIV-$2_{ISY}$ | $SIV_{MAC}$ |
| LTR | 632 | 732 | 800 |
| U3 | 329 | --- | 498 |
| R | 176 | 174 | 176 |
| U5 | 127 | --- | 126 |

**TABLE 15: Sizes of LTR Regions (bps).**
A comparison of sizes of the LTR regions among HIV-2 and SIV retroviruses.

| | gag | pol | vif | vpx | vpr | tat | rev | env | nef |
|---|---|---|---|---|---|---|---|---|---|
| HIV-$2_{ISY}$/HIV-$2_{ROD}$ | 90 | 92 | 86 | 88 | 90 | 81 | --- | 80 | 79 |
| HIV-$2_{NIH-Z}$/HIV-$2_{ROD}$ | 92 | 91 | 86 | 78 | 69 | 85 | 86 | 80 | --- |
| HIV-$2_{NIH-Z}$/$SIV_{MAC}$ | 82 | 75 | 64 | --- | --- | 63 | 59 | 70 | --- |
| HIV-$2_{NIH-Z}$/HIV-1 | 52 | 54 | 28 | --- | 37 | 29 | 34 | 35 | --- |
| $SIV_{MAC}$/HIV-$2_{ROD}$ | 82 | 76 | 64 | --- | --- | --- | --- | 70 | 56 |
| $SIV_{MAC}$/HIV-1 | 51 | 53 | 24 | --- | --- | --- | --- | 34 | 34 |
| HIV-$2_{ROD}$/HIV-1 | 52 | 55 | 28 | --- | --- | --- | --- | 35 | 20 |

--- = Not Done

**TABLE 16: Amino Acid Homologies of HIV-2 and SIV Viral Proteins.**
Percentage of amino acid homology among various HIV-2, HIV-1, and SIV retroviral isolates.

| | HIV-$2_{NIH-Z}$ | HIV-$2_{ROD}$ | $SIV_{MAC}$ | HIV-1 (HTLV-IIIB) | EIAV | VISNA |
|---|---|---|---|---|---|---|
| HIV-$2_{NIH-Z}$ | **100** | 96 | 80 | 67 | 29 | 24 |
| HIV-$2_{ROD}$ | 96 | **100** | 88 | 68 | 28 | 25 |
| $SIV_{MAC}$ | 88 | 88 | **100** | 66 | 29 | 26 |
| HIV-1 (HTLV-IIIB) | 67 | 68 | 66 | **100** | 29 | 26 |
| EIAV | 29 | 28 | 29 | 28 | **100** | 29 |
| VISNA | 24 | 25 | 26 | 26 | 29 | **100** |

**TABLE 17: Amino Acid Homology of the Major gag Proteins (p24/28).**
Percentage of amino acid homology among various retroviral isolates.

| | gag | pol | vif | vpx | env |
|---|---|---|---|---|---|
| HIV-$2_{ISY}$/HIV-$2_{ROD}$ | 11 | 9 | 16 | 13 | 20 |
| HIV-$2_{ISY}$/HIV-$2_{NIH-Z}$ | 12 | 10 | 18 | 19 | 20 |
| HIV-$2_{NIH-Z}$/SIV-$2_{ROD}$ | 8 | 9 | 14 | 17 | 20 |
| HIV-$2_{ISY}$/$SIV_{MAC}$ | 18 | 26 | 37 | 16 | 31 |
| HIV-$2_{NIH-Z}$/$SIV_{MAC}$ | 18 | 25 | 36 | 24 | 30 |
| HIV-$2_{ROD}$/$SIV_{MAC}$ | 13 | 17 | 27 | 15 | 30 |

**TABLE 18: Amino Acid Sequence Divergence of the HIV-2 and SIV Isolates.** Numbers represent percentage of amino acid difference amoung the proteins of the HIV-2 and $SIV_{MAC}$ isolates.

Monkey and Human Sera

| Reactivity to the Synthetic Peptides (PT-1, PT-2) | | | |
|---|---|---|---|
| Species | Number Tested | Number Infected/Uninfected (Western Blot) | Number of Animals Reactive to PT-1 and PT-2 |
| African green monkeys | 17 | 17/0 | 0 |
| Tala poin monkeys | 6 | 3/3 | 0 |
| Macaques (Experimentally infected) | 6 | 3/3 | 2 |
| Baboons (Experimentally infected) | 3 | 3/3 | 0 |
| Humans | 7 | 5/2 | 0 |

**TABLE 19: Monkey and Human Sera Reactivity to the Synthetic Peptides (PT-1 and PT-2).**
Only two macaques out of three experimentally infected monkeys were reactive to peptides PT-1 and PT-2. None of the other monkeys or humans were reactive against these peptides.

| | | Days in Culture | | |
|---|---|---|---|---|
| | | 5 | 8 | 12 |
| MT-2 | % IFA positive | ND | 20 | 32 |
| | RT | 16 | 16 | 29 |
| | Synctia | + | ++ | ++ |
| | % Viable cells | 80 | 61 | 47 |
| CL55 | % IFA positive | ND | 10 | 25 |
| | RT | 6 | 28 | 14 |
| | Synctia | + | ++ | +++ |
| | % Viable cells | 70 | 40 | 36 |
| CEM | % IFA positive | ND | 4 | 18 |
| | RT | 7 | 9 | 12 |
| | Synctia | - | + | + |
| | % Viable cells | 98 | 76 | 71 |
| HUT78 | % IFA positive | 2 | 4 | 18 |
| | RT | 13 | 10 | 36 |
| | Synctia | - | + | ++ |
| | % Viable cells | 90 | 71 | 50 |
| U937 | % IFA positive | 3 | ND | 12 |
| | RT | 8 | 76 | 41 |
| | Synctia | - | ND | ND |
| | % Viable cells | 85 | 78 | 71 |
| H9 | % IFA positive | ND | 10 | 20 |
| | RT | 18 | ND | 90 |
| | Synctia | + | ++ | +++ |
| | % Viable cells | 83 | 63 | 41 |
| MOLT 3 | % IFA positive | ND | 8 | 15 |
| | RT | 9 | 18 | 49 |
| | Synctia | + | ++ | +++ |
| | % Viable cells | 77 | 53 | 43 |

IFA = immunofluorescence assay
RT = cpm x $10^3$
Syncytia = +
% Viable cells = percentage of cells that do not incorporate Trypan blue

**TABLE 20: Infectivity of the HIV-$2_{ISY}$ Isolate.**
HIV-$2_{ISY}$ infected the HTLV0I transformed T-cell line MT-2, the T-cell clone 55 immortalized line, and the CEM, Hut78, Molt3, H9, and U939 neoplastic cell lines. The highest cytopathic effect were observed in the HTLV-I infected cells and in the H9 cells.

| | | Days in Culture | | | |
|---|---|---|---|---|---|
| | | 4 | 7 | 11 | 14 |
| Jurkat | RT | 13 | 12 | 70 | 213 |
| | Synctia | + | ++ | +++ | +++ |
| Hut78 | RT | 3 | 20 | 176 | 155 |
| | Synctia | + | + | + | +++ |
| U937-16 | RT | 17 | 8 | 80 | 279 |
| | Synctia | | | | |

RT = cpm x $10^3$
Syncytia = +

**TABLE 21: Infectivity of the HIV-$2_{SBL6669}$ Viral Isolate.**
The parental virus HIV-$2_{SBL6669}$ infects Hut78, U937 clone 16, and Jurkat T cells. The highest cytopathic effect is observed on the Jurkat and U937-16 cell lines.

| ANIMAL NUMBER | MONTHS AFTER INOCULATION | HIV-$2_{NIH-Z}$ | HIV-$2_{ISY}$ |
|---|---|---|---|
| 172 | 0 (before inoculation) | - | - |
| | 2 | 1:155 | - |
| | 4 | 1:145 | - |
| | 5 | 1:90 | - |
| 176 | 0 | - | - |
| | 2 | - | - |
| | 4 | - | - |
| | 5 | - | - |
| | 7 | - | - |
| 177 | 0 | - | - |
| | 2 | - | - |
| | 4 | - | - |
| | 5 | - | - |
| | 17 | - | - |

**TABLE 22: Neutralizing Antibody Titer in the Infected Rhesus Macaques.** Rhesus macaques were infected with HIV-$2_{NIH-Z}$ (#172) and HIV-$2_{ISY}$ (#176 and #177).

# II. LITERATURE CITED


1. Gallo, R.C. (1987). Sci. Am. **256**, 46-56.

2. Gallo, R. and Montagnier, L. (1988). AIDS in 1988. Sci. Am. **259**, 41-48.

3. Fauci, A.S. (1988). Science **239**, 617-622.

4. Melbye, M., R.J. Biggar, P. Ebbesen, C. Nealand, J.J. Goedert, V. Faber, I. Lorenzen, J.P. Skink, R.C. Gallo, and W.A. Blattner (1986). Ann. Intern. Med. **104**, 496-500.

5. Gottlieb, M.S., R. Schroff, H.M. Schenker, J.D. Weisman, P.T. Fan, R.A. Wolf, and A. Saxon. (1981). N. Engl. J. Med. **305**, 1425-1431.

6. Redfield, R. and D. Burke (1988). HIV Infection: the clinical picture. Sci. Am. **259**, 90-98.

7. McDougal, J., M. Kennedy, J. Sligh, S. Cort, A. Manle, and J. Nicholson (1986). Science **231**, 328-385.

8. Haseltine, W.A. (1988). J. Acquired Immune Deficiency Syndrome **1**, 217-240.

9. Strebel, K., T. Klimkait, and M. Martin (1988). A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science **241**, 1221-1223.

10. Haseltine, W., E. Terwillliger, C. Rosen, and J. Sodroski (1988). in Retrovirus Biology: An emerging role in human diseases, edited by Robert C. Gallo and Flossie Wong-Staal. Marcel Dekker, Inc.

11. Sodroski, J., C. Rosen, F. Wong-Staal, S. Salahuddin, M. Popovic, S. Arya, R.C. Gallo and W. Haseltine (1985). Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. Science **227**, 171-173.

12. Rosen, C. Sodroski, J., and W. Hazeltine (1985). Location of cis acting regulatory sequences in the human T cell lyphotropic virus type 3 (HTLVIII/LAV) long terminal repeat. Cell **41**, 813-823.

13. Fisher, A., L. Ratner, H. Mitsuya, L. Marselle, M. Harper, S. Broder, R.C. Gallo and F. Wong-Staal (1986). Infectious mutants of HTLV-III with changes in the 3' region and markedly reduced cytopathic effects. Science **233**, 655-659.

14. Wong-Staal, F. and R. C. Gallo (1985). Human T-lymphotropic retroviruses. Nature **317**, 395-403.

15. Fisher, A., E. Collati, L. Ratner, R.C. Gallo, and F. Wong-Staal (1985). A molecular clone of HTLV-III with biological activity. Nature **316**, 262-265.

16. Levin and Seidman (1979). J. Virol. **29**, 328-335.

17. Gallis, et al. (1979). Virology **94**, 146-161.

18. Linial, et al. (1978). Cell **15**, 1371-1381.

19. Mann, R. and D. Baltimore (1985). Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNA's. J. Virol. **54**, 401-407.

20. Arya, S.K., Guo, C., Josephs, S.F., Wong-Staal, F., (1985). Transactivator gene of human T-lymphotropic virus type III (HTLV-III). Science **229**, 69-73.

21. Rosen, C.A., et al. (1986). Nature (London) **319**, 555-559.

22. Sodroski, J.G., Goh, W.C., Rosen, C., Dayton, A., Terwillinger, W., Haseltine, W.A. (1986). A second post-transcriptional trans-activator gene required for HTLV-III replication. Nature **321**, 412-417.

23. Jones, K.A., Kadonaga, J.T., Luciw, P.A. and R. Tijian (1986). Science **232**, 755-760.

24. Kanki, P.J., F. Barin, S. M'Boup, J.S. Allan, J.L. Romet-Lemonne, R. Marlink, M.F. McLane, T.H. Lee, B. Arbeille, F. Dennis, and M. Essex (1986). Science **232**, 238-243.

25. Franchini, G., C. Gurgo, R.C. Gallo. E. Collati, H.G. Guo, K.A. Fargnoli, L.F. Hall, F. Wong-Staal, and M.S. Reitz, Jr. (1987). Nature (London) **328**, 539-542.

26. Arya, S.K., and R.C. Gallo (1988). Proc. Natl. Acad. Sci. **85**, 9753-9757.

27. Kong, L.I., S.W. Lee, J.C. Kappes, J.C. Parkin, D. Decker, J.A. Hoaxie, B.H. Hahn, and G.M. Shaw (1988). Science **240**, 1525-1529.

28. Kanki, P., J. Alroy, and M. Essex (1985). Isolation of T-lymphotropic retrovirus related to *HTLV-III/LAV from wild-caught African green monkeys. Science* **230**, 951-954.

29. Zagury, J., G. Franchini, M, Reitz, E. Collati, B. Starcich, L. Hall, K. Fragnoli, L. Jagodzinski, L. Guo, F. Laure, D. Zagury, S. Arya, S. Josephs, F. Wong-Staal, and R.C. Gallo (1988). The genetic variability between HIV-2 isolates is comparable to the variability among HIV-1. Proc. Natl. Acad. Sci. USA . **85**, 5941-5945.

30. Chakrabarti, L., M. Guyader, M. Alizon, M. Daniel, R. Desrosiers, P. Tiollais, and P. Sonigo (1987). Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retrovirus. Nature **328**, 543-547.

31. Symposium on Non-human Primate Models for AIDS. San Antonio, TX. Nov 16-18, 1988.

32. Fisher, A.G., Feinberg, M.B., Josephs, S.F., Harper, M.E., Marselle, L.M., Reyes, G., Gonda, M.A., Aldorini, A., Debouk, C., Gallo, R.C. (1986). The trans-activator gene of HTLV-III is essential for virus replication. Nature **320**, 367-371.

33. Ivanoff, L.A., Towatari, T., Ray, J., Korornt, B.D., and Petteway, S.R., (1986). Expression and site specific mutagenesis of the poliovirus 3C prolease in Escherichia coli. Proc. Natl. Acad. Sci. USA **83**, 5392-5396.

34. Fisher, A., B. Ensoli, L. Ivanoff, M. Chamberlain, S. Petteway, L. Ratner, R.C. Gallo, and F. Wong-Staal (1987). The sor gene of HIV-1 is required for efficient virus transmission in vitro. Science **237**, 888-893.

35. Lee, S-J, Hu, W., Fisher, A.G., Looney, D.J., Kao, V.F., Mitsuya, H., Ratner, L., and Wong-Staal, F. (1989). Role of the Carboxy-Terminal Portion of the HIV-1 Transmembrane Protein in Viral Transmission and Cytopathogenicity. AIDS Research and Human Retroviruses **5**, 441-449.

36. Salahuddin, S.Z. et al., (1985). Proc. Natl. Acad. Sci. USA **82**, 5530-5535.

37. Zagury, D. et al. (1986). Science **231**, 850-853.

38. Harada, S. et al. (1986). Virology **154**, 249-258.

39. Siekevitz, M., S. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. Greene (1987). Activation of the HIV-1 LTR by T-cell mitogens and the tat-1 protein of HTLV-1. Science **238**, 1575-1578.

40. Nabel, G. and D. Baltimore (1987). Nature **326**, 711-713.

41. Sen, R. and D. Baltimore (1986). Cell **46**, 705-716.

42. Sen, R. and D. Baltimore (1986). Cell **47**, 921-928.

43. McDougal, J., et al (1985). J. Immunol. **135**, 3151-3157.

44. Folks, T., et al. (1986). Science **231**, 600-603.

45. Rosen, C.A., J.G. Sodorski, K. Campbell, and W.A. Hazeltine (1986). J. Virol. **57**, 379-387.

46. Ptashne, M. (1988). Nature **335**, 683-689.

47. Meyers, G., S.F. Josephs, J.A. Berzofsky, A.B. Rabson, T.F. Smith, and F. Wong-Staal (1989). Human Retroviruses and AIDS, Los Alamos, NM 11-37.

48. Gorman, C.M., L.F. Moffat, and B.H. Howard (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell Biol. **2**, 1044-1051.

49. Seigel, L.J., Ratner, L., Josephs, S.F., Derse, D., Feinberg, M.B., Reyes, G.R., O'Brien, S.J., Wong-Staal, F. (1986). Transactivation induced by human T-lymphotropic virus type III (HTLV-III) maps to a viral sequence encoding 58 amino acids and lacks tissue specificity. Virology **148**, 226-231.

50. Knight, D.M., F.A. Flomerfelt, and J. Gharayeb (1987). Science **236**, 837-840.

51. Zoller, M.J., and M. Smith (1983). Methods Enzymol **100**, 468-500.

52. Tooze, J. in The Molecular Biology of Tumor Viruses. (1973). Cold Spring Harbor Press, New York.

53. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., Schaffner, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell **41**, 521-530.

54. Nitta, M. et al. (1985). Blood **66**, 1053-1061.

55. Rosenfeld, P. and T. Kelly (1986). J. Biol. Chem. **261**, 1398-1408.

56. Inoue, J. et al. (1986). WMBO J. **5**, 2882.

57. Maruyama, M. et al. (1987). Cell **48**, 343-348.

58. Cross, S.L. et al. (1987). Cell **49**, 47-52.

59. Starcich, B. et al. (1985). Science **227**, 538-542.

60. Cullen, B.R. (1986). Cell **46**, 973-982.

61. Wright, C., Felber, B., Paskalis, H., and Pavlakis, G. (1986). Science **234**, 988-992.

62. Heisig, V., T. Benter, S.F. Josephs, M.R. Sadaie, T. Okamoto, R.C. Gallo, and F. Wong-Staal. In Neth., R., R.C. Gallo, M.F. Greaves, and H. Habish (Eds.): Modern Trends in Human Leukemia VII. Berlin, Springer Verlag (1987). **31**, 423-429.

63. Franza B.R., Jr., Josephs, S.F., Gilman, M.Z., Ryan, W., & Clarkson, B. (1987). Characterization of Cellular Proteins Recognizing the HIV Enhancer Using a Microscale DNA-Affinity Precipitation Assay. Nature **330**, 391-395.

64. Chou, P.Y. and G.D. Fasman (1974). Biochemistry **13**, 222-245.

65. Giniger, E. and M. Ptashne (1987). Nature **330**, 670-672.

66. Gendelman, H.E. et al. (1986). Proc. Natl. Acad. Sci. USA **83**, 9759-9763.

67. Robert-Guroff, M. et al. (1986). J. Am. Med. Assoc. **255**, 3133-3139.

68. Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Brun-Yezinet, F., Rouzioux, C., Rozenbaum, W., Montagnier, L. (1983). Science **220**, 868-871.

69. Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M.-A., Santos-Ferreria, M.O., Laurent, A.G., Dauguet, C., Katlama, C., Rouzioux, C., Klatzmann, D., Champalimaud, J., Montagnier, L. (1986). Science **233**, 343-346.

70. Clavel, F., Mansinho, K., Chamaret, S., Guetard, D., Favier, V., Nina, J., Santos-Ferreira, M.O., Champalimaur, J.L., Montagnier, L. (1987). N. Engl. J. Med. **316**. 1180-1185.

71. Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleske, J., Safai, B., White, G., Foster, P., Markham, P.D. (1984). Science **224**, 500-503.

72. Popovic, M., Sarngadharan, M.G., Read, E., Gallo, R.C. (1984). Science **224**, 497-500.

73. Sarngadharan, M.G., Popovic, M., Bruch, L., Schupbach, J., Gallo, R.C. (1984). Science **224**, 506-508.

74. Albert, J., Bredberg, U., Chiodi, P., Bottiger, B., Fenyo, E.M., Norrby, E. and Biberfeld, G. (1987). AIDS Res. Hum. Retroviruses **3**, 1-10.

75. Daniel, M.D., Letvin, N.L., King, N.W., Kannagi, M., Sehgal, P.K., Hunt, R.D., Kanki, P.J., Essex, M., Desrosiers, R.C. (1985). Science **228**, 1201-1204.

76. Benveniste, R.E., Arthur, L.O., Tsai, C.-C., Sowder, R., Copeland, T.D., Henderson, L.E., Oroszlan, S. (1986). J. Virol. **60**, 483-490.

77. Fultz, P.N., McClure, H.M., Anderson, D.C., Brent-Swenson, R.B., Anand, R., Srinivasan, A. (1986). Proc. Natl. Acad. Sci. USA **83**, 5286-5290.

78. Murphey-Corb, M., Martin, L.N., Rangan, S.R.S., Bashin, G.B., Gormus, B.J., Wolf, R.H., Andes, W.A., West, M., Montellaro, R.C. (1986). Nature (London) **321**, 435-437.

79. Fukasawa, M., Miura, T., Hasegawa, S., Morikawa, S., Tsujimoto, H., Miki, K., Kitamura, T., Hayami, M. (1988). Nature **333**, 457-461.

80. Hirsch, V.M., Olmsted, R.A., Murphey-Corb, M., Purcell, R.H., Johnson, P.R. (1989). Nature **339**, 389-392.

81. Feinberg, M.B., Jarrett, R.F., Aldovini, A., Gallo, R.C., Wong-Staal, F. (1986). Cell **46**, 807-817.

82. Wong-Staal, F.,Chandon, P.K., Ghrayeb, J. (1987). AIDS Res. Hum. Retroviruses **3**, 33-40.

83. Franchini, G., Rusche, J. R., O'Keeffe,T. J. & Wong-Staal, F. (1988). AIDS Res. Hum. Retroviruses **4**, 243-250.

84. Henderson, L.E., Sowder, R.C., Copeland, T.D., Benveniste, R.E., Oroszlan, S. (1988). Science **24**, 199-201.

85. Sambrook. J., Fritsch, E.F., Maniatis, T. (1989). Molecular Cloning, A Laboratory Manual. Cold Spring Harbor, Volumes 1-3.

86. Franchini, G., Collatti, E., Arya, S.K., Fenyo, E.M., Biberfeld, G., Zagury, J.F., Kanki, P.J., Wong-Staal, F., Gallo, R.C. (1987). AIDS Res. Hum. Retroviruses **3**, 11-17.

87. Hanahan, D. (1985). DNA Cloning, Vol. 1 (ed. D.M. Glover) IRL Press. A Practical Approach **6**, 106-135.

88. Southern, E.M. (1975). J. Mol. Biol. **98**, 503-517.

89. Sanger, F., Nicklen, S., Coulson, A.R. (1977). Proc. Natl. Acad. Sci. USA **74**, 5463-5467.

90. Maxam, A., and Gilbert, W. (1977). Proc. Natl. Acad. Sci. USA **74**, 560-563.

91. Rosenberg, N. and Baltimore, D. (1978). J. Exp. Med **147**, 1126-1141.

92. Harlow, E. and Lane, D. (1988). Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory.

93. Biberfeld, P., Kramarsky, B., Salahuddin, S.Z., Gallo, R.C. (1987). J. Natl. Cancer Inst. **79**, 933-941.

94. Towbin, H.H., Staehelin, T., Gordon, J. (1979). Proc. Natl. Acad. Sci USA **76**, 4350-4356.

95. Polez, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, S.D., Gallo, R.C. (1980). Science **220**, 450-458.

96. Laemmli, U. (1970). Nature **227**, 680-685.

97. Kappes, J.C., Morrow, C.D., Lee, S.W., Jameson, B.A., Kent, S.B.H., Hood, L.E., Shaw, G.M., Hahn, B.H. (1988). J. Virol. **62**, 3501-3505.

98. Cohen, E.A., Terwilliger, E.F., Sodroski, J.G. and Haseltine, W.A. (1988). Nature (London) **334**, 532-534.

99. Ratner, L., Haseltine, W., Paturea, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Antoni Rafalski, J., Whitehorn, E.A., Baumeister, K., Ivanoff, L, Petteway, S.R., Pearson, M.L., Lautenberg, J.A., Papas, T.K., Ghrayeb, J., Chang, N.T., Gallo, R.C., and Wong-Staal, F. (1985). Complete nucleotide sequence of the AIDS virus HTLV-III. Nature **313**, 277-284.

100. Dalgleish, A.G., Beverly, P.C.L., Clapham, P.R., Crawford, D.H., Greaves, M.F., and Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312**, 763-767.

101. Klatzmann, D., Champagne, E., Charmaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J., And Montagnier, L. (1984). T-Lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature **312**, 767-771.

102. Kornfeld, H., Riedel, N., Viglianti, A., Hirsch, V., and Mullins, J.I. (1987). Cloning of HTLV-4 and its relation to simian and human immunodeficiency viruses. Nature **326**, 610-613.

103. Cease, K.B., Margalit, H., Cornette, J.L., Putney, S.D., Robey, W.G., Ouyang, C., Streicher, H.Z., Fischinger, P.J., Gallo, R.C., DeLisi, C., and Berzofsky, J.A. (1987). Helper T-cell antigenic site identification in the acquired immunodeficiency syndrome virus gp120 envelope protein and induction of immunity in mice to the native protein using a 16-residue synthetic peptide. Proc. Nat. Acad. Sci. USA **84**, 4249-4253.

104. Modrow, S., Hahn, B.H., Shaw, G.M., Gallo, R.C., Wong-Staal, F., and Wolf, H. (1987). Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J. Virol. **61**, 570-578.

105. Gallaher, W.R. (1987). Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell **50**, 327-328.

106. Sodroski, J., Goh, W.C., Rosen, C., Tartar, A., Portelle, D., Burny, A., Haseltine, W., (1986). Replicate and cytopathic potential of HTLV-III/LAV with sor gene deletions. Science **231**, 1549-1553.

107. Hirsch, V., Riedel, N., Mullins, J.I. (1987). The genome organization of STLV-3 is similar to that of the AIDS virus except for a truncated transmembrane protein. Cell **49**, 307-309.

108. Starcich, B.R., Hahn, B.H., Shaw, G.M., McNeehy, P.D., Modrow, S., Wolf, H., Parks, W.P., Josephs, S.F., Gallo, R.C., Wong-Staal, F. (1986). Cell **45**, 637-648.

109. Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A., and Wain-Hobson, S. (1985). Nucleotide sequence of the Visna lentivirus: Relationship to the AIDS virus. Cell **42**, 369-382.

110. Allan, J.S., Coligan, J.E., McLane, M.F. (1985). Science **230**, 810-813.

111. Aldovini, A., Debouck, C., Rosenberg, M., Arya, S., and Wong-Staal, F. (1987). Expression of the complete transactivator gene product of HTLV-III (AIDS Virus) in E. coli: Its immune reactivity with sera from HTLV-III antibody positive individuals. Proc. Nat. Acad. Sci. USA.

112. Arya, S.K., Beaver, B., Jagodzinski, L., Ensoli, B., Kanki, P.J., Albert, J., Fenyo, E., Biberfeld, G., Zagury, J.F., Laure, F., Essex, M., Norrby, E., Wong-Staal, F., and Gallo, R.C. (1987). New human and simian HIV related retroviruses possess functional transactivator (tat) gene. Nature **328**, 548-550.

113. Ratner, L., Starcich, B., Josephs, S., Hahn, B., Reddy, E., Livak, K., Petteway, S., Pearson, M., Haseltine, W., Arya, S.K., and Wong-Staal, F. (1985). Polymorphism of the 3' open reading frame and long terminal repeat sequences of the virus associated with the acquired immunodeficiency syndrome, human T-lymphotropic virus type III. Nucleic Acids Research **13**, 219-229.

114. Franchini, G., Fragnoli, K.A., Giombini, F., Jagodzinski, L., DeRossi, A., Bosch, M., Biberfeld, G., Fenyo, E.M., Alberts, J., Gallo, R.C., Wong-Staal, F. (1989) Proc. Natl. Acad. Sci USA **86**, 2433-2437.

115. Franchini, G., Kanki, P. J., Bosch, M. & Fargnoli, K. A. (1988). AIDS Res. Hum.Retroviruses **4**, 251-258.

116. Aldovini, A., DeRossi, A., Feinberg, M., Wong-Staal, F. & Franchini, G. (1986). Proc. Natl. Acad. Sci. USA, 38-42.

117. Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., Fischinger, P.J. (1987) AIDS Research and Human Retroviruses **3**, 283-302.

118. Saxinger, W.C., Levine, P.H., Dean, A.G., De-the, G., Moghissi, J., Jaurent, F., Hoh, M., Sarngadharan, M.G., and Gallo, R.C. (1985). Evidence for exposure to HTLV-III in Uganda prior to 1973. Science **227**, 1036-1038.

119. Franchini, G., Robert-Guroff, M., Wong-Staal, F., Ghrayeb, J., Katz, I., Chang, T.W. and Chang, N.T. (1986). Expression of the protein encoded by the 3' open reading frame of HTLV-III in bacteria: Demonstration of its immunoreactivity with human sera. Proc. Natl. Acad. Sci. **83**, 5282-5285.

120. Kanki, P.J., Barin, F., M'Boup, S., Allan, J.S., Romet-Lemonne, J.L., Marlink, R., McLane, M.F., Lee, T.H., Arbeille, B., Dennis, F., and Essex, M. (1986). Science **232**, 238-243.

121. Kanki, M.J., M'Boup, S., Ricard, D., Barin, F., Denis, F., Boye, C., Sangare, L., Travers, K., Albaum, M., Marlink, R., Romet-Lemonne, J.L., and Essex, M. (1987). Human T-lymphotropic virus type 4 and the human immunodeficiency virus in West Africa. Science **236**, 827-831.

122. Arya, S.K. (1988). AIDS Res. Hum. Retroviruses **4**, 175-186.

123. Colombini, S., Arya, S.K., Reitz, M.S., Jagodzinski, L., Beaver, B. and Wong-Staal, F. (1989). Structure of SIV regulatory genes. Proc. Natl. Acad. Sci. USA , **86**, 4813-4817

124. Alter, J. H., Eichberg, J. W., Masur, H., Saxinger, W.C., Gallo, R. C., Macher, A. M., Lane, H. C. and Fauci, A. S. (1984). Science **226**, 549-552.

125. Markham, P. and Franchini, G. Personal communication.

126. Looney, D.J., Fisher, A.G., Putney, S.D., Rosche, J.R., Redfield, R.R., Burke, D.S., Gallo. R.C., Wong-Staal, F. (1988). Science **241**, 357-359.

127. Hahn, B.H., Shaw, G.M., Taylor, M.E., Redfield, R.R., Markham, P.D., Salahuddin, S.Z., Wong-Staal, F., Gallo, R.C., Parks, E.S., Parks, W.P. (1986). Science **232**, 1548-1553.

128. Meyerhans, A., Cheynier, R., Albert, J., Seth, M., Kwok, S., Sninsky, J., Morfeldt-Manson, L., Asjo, B., Wain-Hobson, S. (1989). Cell **58**, 901-910.

129. Naidu, Y.M., Kestler III, H.W., Li, Y., Butler, C.U., Silva, D.P., Schmidt, D.K., Troup, C.D. Sehgal, P.K., Sonigo, P., Daniel, M.D., Desrosiers, R.C. (1988). J. Virol. **62**, 4691 4696.

130. Kestler, H., Kodama, T., Ringler, D., Marthas, M., Dedersen, N., Lackner, A., Regier, D., Sehgal, P., Daniel, M., King, N., Desrosiers, R.C. (1990). Science **248**, 1109-1112.

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# IV. PUBLICATIONS ARISING FROM CONTRACT


Franchini, G., Markham, P., Gard, E., Fargnoli, K., Keubaruwa, S., Jagodzinski, L., Robert-Guroff, M., Lusso, P., Ford, G., Wong-Staal, F., Gallo, R.C.: Persistent Infection of Rhesus Macaques with a Molecular Clone of HIV-2: Evidence of Minimal Genetic Drift and Pathogenetic Effects. J. Virology 64:4462-4467, 1990.

Colombini, S., Arya, S.K., Reitz, M.S., Jagodzinski, L., Beaver, B., Wong-Staal, F.: Structure of simian immunodeficiency virus regulatory genes. Proc. Natl. Acad. Sci. 86:4813-4817, 1989.

Franchini, G., Fargnoli, K.A., Giombini, F., Jagodzinski, L., De Rossi, A., Bosch, M., Biberfeld, G., Fenyo, E.M., Albert, J., Gallo, R.C., Wong-Staal, F.: Molecular and Biological Characterization of a Replication Competent Human Immunodeficiency Type-2 (HIV-2) Proviral Clone. Proc. Natl. Acad. Sci. 86, 2433-2437, 1989.

Lee, S-J, Hu, W., Fisher, A.G., Looney, D.J., Kao, V.F., Mitsuya, H., Ratner, L., and Wong-Staal, F. Role of the Carboxy-Terminal Portion of the HIV-1 Transmembrane Protein in Viral Transmission and Cytopathogenicity. AIDS Research and Human Retroviruses, Vol 5, pp. 441-449, 1989.

Rappaport, J., Josephs, S.F., Klotman, M.E., Kang, C.Y., Almeda, S., Daefler, S., Frankel, A.D., Rusche, J.R., and Wong-Staal, F. HIV-1 Tat Binds to the 5' Region of Viral mRNA. Submitted 1989.

Franchini, G., Fargnoli, K., Giombini, F., O'Keeffe, T., et al. Identification of the HIV-2 X Protein and Study of Its Function in a Biologically Active Genome of HIV-2 (Meeting Abstract). Fourth International Conference on AIDS. Book II., 1988.

Franchini, G., Kanki, P.J., Bosch, M.L., Fargnoli, K., and Wong-Staal, F. The Simian Immunodeficiency Virus Envelope Open Reading Frame Located After the Termination Codon Is Expressed In Vivo in Infected Animals. AIDS Research and Human Retroviruses. Vol. 4, No. 4, pp. 251-258, 1988.

Franchini, G., Rusche, J.R., O'Keeffe, T.J., and Wong-Staal, F. The Human Immunodeficiency Virus Type 2 (HIV-2) Contains a Novel Gene Encoding a 16kD Protein Associated with Mature Virions. AIDS Research and Human Retroviruses. Vol. 4, No. 4, pp. 243-250, 1988.

Looney, D.J., Fisher, A.G., Putney, S.D., Rusche, J.R., Redfield, R.R., Burke, D.S., Gallo, R.C., Wong-Staal F. Type-Restricted Neutralization of Molecular Clones of Human Immunodeficiency Virus. Science, Vol. 241, pp. 357-359, 1988.

Sadaie, M.R., Josephs S.F., Rappaport J., Willis R., et al. Functional Analysis of the tat and trs Genes of Human Immunodeficiency Virus by Site-Directed Mutagenesis. Vaccines 88: New Chemical and Genetic Approaches to Vaccination, 1988.

Sadaie, M.R., Rappaport, J., Benter, T., Josephs, S.F., Willis, R., and Wong-Staal, F. Missense mutations in an infectious human immunodeficiency viral genome: Functional mapping of *tat* and identification of the *rev* splice acceptor. Proc. Natl. Acad. Sci. USA. Vol. 85, pp. 9224-9228, 1988.

Zagury, J.F., Franchini, G., Reitz, M., Collalti, E., Starcich, B., Hall, L., Fargnoli, K., Jagodzinski, L., Guo, H.-G., Laure, F., Arya, S.K., Josephs, S., Zagury, D., Wong-Staal, F., and Gallo, R.C. Genetic Variability Between Isolates of Human Immunodeficiency Virus (HIV) Type 2 is Comparable to the Variability Among HIV Type 1. Proc. Natl. Acad. Sci. USA. Vol. 85, pp. 5941-5945, 1988.

Fisher, A., Ensoli, B., Ivanoff, L., Chamberlain, M., Petteway, S., Ratner, L., Gallo, R.C., and Wong-Staal, F. Science 237, 888-893, 1987.

Franchini, G., Gurgo C., Guo, H.-G., Gallo, R.C., Collalti, E., Fargnoli, K.A., Hall, L.F., Wong-Staal, F., and Reitz Jr., M.S. Sequence of Simian Immunodeficiency Virus and Its Relationship to the Human Immunodeficiency Viruses. Nature, Vol. 328, pp. 539-543, 1987.

Franchini, G., Gurgo, C., Gallo, R.C., Collati, E., Guo, H.G., Fargnoli, K.A., Hall, L.F., Wong-Staal, F., and Reitz, M.S. Jr. Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. Nature (London) 328, 539-542, 1987.

Franza B.R., Jr., Josephs, S.F., Gilman, M.Z., Ryan, W., & Clarkson, B. Characterization of Cellular Proteins Recognizing the HIV Enhancer Using a Microscale DNA-Affinity Precipitation Assay. Nature, Vol. 330, pp. 391-395, 1987.

Heisig, V., Benter, T., Josephs, S.F., Sadaie, M.R., Okamoto, T., Gallo, R.C., and Wong-Staal, F. Interaction of Viral and Cellular Factors with the HTLV-III LTR Target Sequences In Vitro. Haematology and Blood Transfusion, Vol. 31, pp. 423-429, 1987.

Siekevitz, M., Josephs, S.F., Dukovich, M., Peffer, N., Wong-Staal, F., Greene, W.C. Activation of the HIV-1 LTR by T Cell Mitogens and the Trans-Activator Protein of HTLV-I. Science, Vol. 238, pp. 1575-1578, 1987.

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